UNIVERSITY OF BIRMINGHAM

IN-VITRO DIGESTIVE PROCESSES OF STRUCTURED FOODS

by

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ABSTRACT

Since the human gastrointestinal tract is a complicated system, understanding how food is digested requires appropriate methods. An *in-vitro* approach offers a reliable method to study food digestion process. This study will outline the utilisation of in-vitro digestive models on the digestion of structured food focusing on rice and low acyl gellan gum. Understanding the behaviour of rice and low acyl gellan gum in the digestive system is essential to tailor healthier staple food products with desired characteristics.

In the initial experiment we used *in-vitro* static and dynamic models including Infogest static digestion protocols, stirred tank reactor and dynamic duodenum model to the samples ranging from the simplest drug model, glucose solution, semi-solid food (bread and rice) and solid grain matrices. All the methods were suitable to evaluate real food matrices such as bread and cooked rice.

The present study on incorporating rice and gellan gum to create slow digestible cooked rice have been considered. The Infogest static method and multi-scale analysis were used to evaluate its digestion proses. The research has shown that adding 1% low acyl gellan gum to rice cooking could reduce the GI of white rice in *in-vitro* experiments by 16%. The role of low acyl gellan gum in protecting the rice surface from the penetration of intestinal enzymes and fluids can be explained in the multi-scale analysis.

STR models provided a straightforward approach to evaluate the effect of mixing and calculating mass transfer from the permeable walled cylinder tank to the water outside of the membrane. The same aim with STR, DDM enables mechanical compression, similar to that found in the human gut system, that can be set to run contraction and peristaltic movement. On average, STR had the highest rate of digestibility, and the lowest was DDM without contraction. The use of the 2 cpm mode improved the digestibility of rice by 30%.

Overall, structuring cooked rice using additional low acyl gellan gum created low digestible rice which will benefit as a healthier staple food for people with diabetes and obesity. The *in-vitro* digestion examination by Infogest static method, STR and DDM show noticeable results in this study.

ACKNOWLEDGEMENT

Finishing this piece of work is a relieve through-out up and down, especially in the final year when Covid-19 pandemic hit all of us. I could not express my gratefulness to Almighty God, Allah SWT, with His mercy and guide so I can pass this chapter of life, Alhamdulillah. Furthermore, a great blessing to our prophet Muhammad, may peace be upon him, how beautiful is your guidance.

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ABBREVIATIONS

Cryo-SEM	Cryo Scanning Electron Microscopy
DDM	Dynamic Duodenum Model
ESEM	Environmental Scanning Electron Microscopy
GI	Glycaemic Index
HAG	High Acyl Gellan Gum
HSA	Human Salivary Amylase
LAG	Low Acyl Gellan Gum
MRI	Magnetic Resonance Imaging
PAHBAH	4-Hydroxybenzhydrazide
RC	Oral processed rice using mini chopper
RDS	Rapid Digestible Starch
RG	Cooked Rice Grain
RM	Oral processed rice by mouth mastication
RS	Resistant Starch
SDS	Slow Digestible Starch
SEM	Scanning Electron Microscopy
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SSF	Simulated Salivary Fluid
STR	Stirred Tank Reactor

NOMENCLATURES

A	Surface area (m^2)
°C	Degree Celcius
cpm	contraction per minute
C	Concentration
ΔC	Concentration difference (mol m^{-3})
H	Height
K	Mass Transfer Coefficient
L	Length
M	Molar
min	minute
mL	Mililiter
mM	Milimolar
mm	Milimeter
$Vmax$	Maximum velocity or rate
U/ml	Unit per mililiter
µM	Micromolar
µL	Microliter
S	substrate concentration
So	initial substrat concentration
Km	Michaelis half saturation constant
Y_0	value of Y when X (time) is zero
$Plateau$	value of Y at infinite intervals
$KFast$	rate constant fast
$KSlow$	rate constant slow
D	diameter (m)
$mol_{maltose}$	maltose in the recipient side (mol)
M_T	the total molar flux (mol $m^{-2}s^{-1}$)

CHAPTER 1

Introduction

1.1. Overview

The World Health Organization published that the world population suffering from obesity has tripled for the past three decades. In 2016, nearly 2 billion of adults were overweight, and one-third of them were obese (WHO, 2018). In line with this, more than quarter of UK adults citizen were obese in 2014 and will increase to nearly 35% by 2025. The snowball effect of this condition is in the UK there will be a massive rise in the cost of treating illness caused by obesity from $19 billion to $34 billion per year by 2025 (The Guardian, 2018). This number is not surprising if we step forward to the increases of a wide range chronic disease induced by being overweight, such as depression, stroke, type 2 diabetes and even some cancers.

Actually, obesity is a preventable health problem, mainly by sustained healthy diet and lifestyle. However, at present, busy lifestyles require people to consume easy-to-cook and highly processed food. These kinds of meals lead people to consume unhealthy fat and easy to digest carbohydrates in their diet. This phenomenon provokes the food industry to develop novel healthy meals with structural and functional property to prevent obesity.

Some of the attempts to reduce caloric content of the foods are using additional dietary fibre, modified starch, artificial or natural sweetener as well as adjusting the structure of foods. A study of dietary fibre function in the upper human gut come to a

conclusion that dietary fibre could promote gastric emptying then affecting satiety (Mackie, Bajka and Rigby, 2016) Controlling nutrient rate release of processed food could be done by modifying its structure using the addition of hydrocolloid materials such as pectin, carrageenan, gellan gum, guar gum and so on.

In line with this effort, understanding the digestion mechanism of specifically formulated food is a trend among scientists. For instance, understanding the mechanism of rice digestion in the human gut may lead to a possibility of creating a rice product with a lower glycaemic response. Chen et al. (2017) have succefully added pullulan on rice starch resulting in significant increase of slowly digestible starch and deceleration of hydrolysis rate.

Due to the complexity of the human digestive tract, understanding the food digestion process with proper application methods is essential. Up to the present, the studies of food digestion can be done by three type of methodologies, named *in-vivo*, *in-vitro* and *in-silico*. The *in-vivo* experiments use animal or human trials which are costly and need ethical approved. The food digestion studies which are conducted outside the body are called *in-vitro* methods. These laboratory experiments are cost-effective but more consideration must be taken to make sure it's replicable and representative of a real digestion processes. The *in-silico* method involves a mathematical model and computational methodologies.

The *in-vivo* study is facing a slow development because of costly experiments, current issue of animal protection and ethical aspect of human involved tests. This situation makes an *in-vitro* approach as the most reliable method to study food digestion processes.

This study will outline the utilisation of *in-vitro* digestive models on the digestion of structured food. The thesis includes preliminary work results as fundamental for further experiment and to understand the mechanism of the models. The main study of the structured food digestion will be focused on rice and the addition of gellan gum by using *in-vitro* static and dynamic models.

1.2. Objectives

This research has focused on applying *in-vitro* models to understand how the digestion of structured food and its additional hydrocolloids behave in the digestive system. The specific objectives are as the following list:

a) To understand the application of *in-vitro* digestion models including Infogest static methods, stirred tank reactor (STR) and dynamic duodenum model (DDM) on the food matrices.

b) To examine the rice digestibility with and without additional hydrocolloid and understand its effect using a multi-scale approach.

c) To study the effect of additional low acyl gellan gum on rice digestion while evaluating mixing and mass transfer process using STR and DDM.

1.3. Thesis Layout Overview

This thesis is presented in 6 chapters which cover the background and objectives of the study, development of the methods and main study on the in-vitro rice digestion. Then the final part is the end of each experiment and potential study to expand the information gained from the current work.

The thesis contains a general introduction to the area of in-vitro digestion, while the main experimental chapters are presented in the format of scientific journal paper. The content of each chapter is summarized as below:

Chapter 1 : Introduction

A description of background that leads to research study, objectives of the study and overview of the thesis outline.

Chapter 2: Literature Review

The chapter provides some descriptions of fundamental knowledge on the human digestion system, current methods and techniques for mimicking food processing in gastrointestinal tract and basic theory of glycaemic index. Since food composition and structure plays important roles in the digestive system, this chapter presents basic aspect of carbohydrate, dietary fiber and hydrocolloids. The other part is related with the information of starch base food materials especially rice as a research object.

Chapter 3 : Developing Methods

A list of methods that have been conducted as preliminary works on this study. This will include laboratory practical work which is adopted and modified from the previous study as well as develop the method for the present study. This chapter includes 3 sections:

- Section 3.1. is about developing gastric models to understand the effect of dilution on nutrient absorption.
- Section 3.2. presents preliminary work on the Dynamic Duodenum Model (DDM) using simple glucose and hydrocolloid solution
- Section 3.3. covers experimental work on *in-vitro* food digestion using Infogest static methods.
- Section 3.4 provides some experimental results of bread digestibility using in-vitro DDM
- Section 3.5 introduces bolus disintegration experiment using 'food-on-stick' chamber

Chapter 4: Reducing Starch Digestion of White Rice by Structuring with Hydrocolloids

This chapter describes the effect of additional hydrocolloids, focusing low acyl gellan gum on the digestion process of cooked rice. The study aimed to investigate how low acyl gellan gum could lower rice digestibility. The paper includes a multi-scale study of rice structure in the in-vitro digestion process.

Chapter 5 : In-vitro Digestion of rice with additional low acyl gellan gum using static and dynamic models

The chapter presents the comparison of practical in-vitro digestion model on the rice digestion using Infogest static method and Dynamic Duodenum Models. The chapter also includes a pilot study of MRI study of rice in the stomach. However, the experiment has been forced to be postponed as the impact of Covid19 pandemic and could not be presented in this thesis. The experiment was a collaborative study with Medical School of University of Nottingham.

Chapter 6 : Conclusion

The chapter summarizes founding in in-vitro digestion using static and dynamic models mainly on the rice digestibility by adding low acyl gellan gum. The last part includes suggesting future works that may further impact the developing food material that has slower digestibility.

1.4. Publications and Conferences

Part of these works has been published and presented in the various forums listed below.

Publications:

a. Muttakin, S.; Moxon, T. E. and Gouseti, O. 2019. *In-vivo*, *in-vitro*, and *in-silico* studies of the GI tract. *Interdisciplinary Approaches to Food Digestion*. Book Chapter. doi: 10.1007/978-3-030-03901-1_3.

b. Malik, N.; Muttakin, S.; Lopez-Quiroga, E.; Watson, N.J.; Fryer, P.J.; Bakalis, S. (2020). Microstructure and reconstitution of freeze-dried gum Arabic at a range of concentrations and primary drying temperatures. *Food Hydrocolloids*. Elsevier Ltd, 104 (September 2019), p. 105712. doi: 10.1016/j.foodhyd.2020.105712.

c. Kristensen, K.; David-rogeat, Noemie; Alsammari, Norah; Liu, Qingsu; Muleya, Molly; **Muttakin, Syahrizal**; Marciani, Luca; Bakalis, Serafim; Foster, Tim J. and Gouseti, Ourania. (2019) *Chapter 10 - Food Digestion Engineering*, Sustainable Food Processing and Engineering Challenges. Elsevier Inc. doi: 10.1016/B978-0-12-822714-5/00010-3.

d. Alshammari, N.A.; Taylor, M.A.; Stevenson, R.; Gouseti, O.; Alyami, J.; **Muttakin, S**.; Bakalis, S.; Lovegrove, A.; Aithal, G.P.; Marciani, L. (2021). Effect of Intake of Food Hydrocolloids of Bacterial Origin on the Glycemic Response in Humans: Systematic Review and Narrative Synthesis. *Nutrients*., 13, 2407. https://doi.org/10.3390/nu13072407

e. Oliveira, L.C.; Macnaughtan, B.; Gouseti, O.; Villas-Boas, F.; Clerici, M.T.P.S.; Bakalis, S.; **Muttakin, S**. and Cristianini, M. (2021). Extending the functionality of arrowroot starch by thermally assisted high hydrostatic pressure. *Journal of Food Processing and Preservation.* Wileys. DOI: 10.1111/jfpp.15756.

Conferences:

a. **EFFoST** (The European Federation of Food Science and Technology). 32nd EFFoST International Conference , Nantes, Frances, 5 - 8 November 2018. *Improving White Rice Digestibility by Addition of Gellan Gum: An In-vitro Study*. Syahrizal Muttakin, Ourania Gouseti, Cathrina Edwards, Peter Fryer and Serafim Bakalis.
b. **INFOGEST**. 6th International Conference on Food Digestion, Granada, Spain, 2 – 4 April 2019. *Changing of structural and digestion behaviour of white rice by the addition of hydrocolloids.* Syahrizal Muttakin, Alexander Lammond, Ourania Gouseti, Peter Fryer and Serafim Bakalis.
c. **EFFoST** (The European Federation of Food Science and Technology). 33rd EFFoST International Conference, Rotterdam, The Netherlands, 12 - 14 November 2019. *Engineer Digestion of Rice With Hydrocolloids by Exploring The Functionality of Hydrocolloids.* Syahrizal Muttakin, Alexander Lammond, Ourania Gouseti, Peter Fryer and Serafim Bakalis.

CHAPTER 2

Literature Review

2.1. Human Digestive System

Digestion is a crucial process in the body that occurs in the gastrointestinal tract (GIT) and is responsible for breaking down ingested food into nutrients and waste. The digestion tract is divided into 4 distinctive stages; oral processing, gastric phase, small intestine phase and large intestine phase. Figure 1 displayed each digestion phase with the description and associate engineering solution.

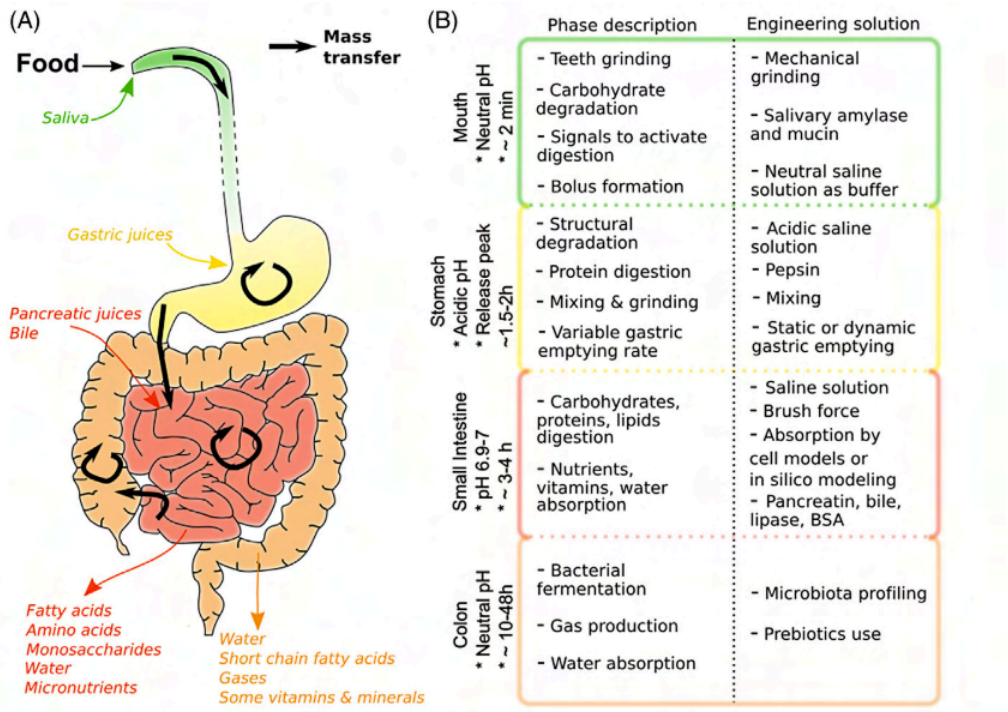


Figure 1. The human digestive system, with its four main parts colored differently (A), and their function in food digestion, along with the associated engineering solution (B). The thin arrows pointing inward represent the addition of fluid by supporting organs, while the arrows pointing outward represent absorption by the small and large intestines. The thick arrows indicate mass transfer down the digestive system and inside the digestive organs. Taken from Kristensen *et al.* (2021)

The oral phase reduces food particle size using mechanical force and salivary enzyme to form a bolus. Then the bolus moves to the gastric phase, in which food is stored where some chemical and enzymatic breakdown occur. In the small intestine, the chyme is continuously broken down, and nutrients are absorbed. The final stage of food digestion happens in the large intestine, during which water is eliminated, fermentation occurs, and waste material is expelled (Jennifer E. Norton *et al.*, 2014).

As the initial phase in food digestion, oral digestion involves two simultaneous actions: chewing to provide mechanical grinding and enzymatic starch hydrolysis, which is mediated by saliva, to break down starch. From the engineering perspective, the oral phase can be mimicked using mechanical force to reduce the particle size of the food and simulated salivary fluid with addition salivary amylase or alpha-amylase to degrade starch (Kristensen *et al.*, 2021). The salivary amylase could hydrolyse starch for around 80% bread starch and 30% pasta starch (Freitas and Le Feunteun, 2019).

Bolus from oral processing is transferred to the gastric phase, where the food is kept in the acidic environment, pH around 2-3. In this stage, protein digestion happens with the help of the digestive enzyme. Bolus is mixed and ground to form a smaller particle size of food matrice, named chyme, containing solid particles and liquid phase. The digesta is experiences a strong peristaltic waves and stomach acts as sieve, allowing the smaller particles to move quicker heading to small intestine (Kristensen *et al.*, 2021).

In-vitro digestion is trying to simulate the gastric phase process. The engineering solutions for food digestion in the stomach are mixing bolus with acidic simulated gastric fluid with addition pepsin, mechanical action to mimic peristaltic movement, and monitoring gastric emptying with model food/bolus (Muttakin, Moxon and Gouseti, 2019).

Chyme from gastric phase travels to the small intestine phase and is mixed with bile and pancreatic secretion containing enzymes, including a-amylase and protease. In the small intestine, carbohydrate, protein and lipids digestion is continuously occurred. The digestion process is triggered by the two movement pattern, defined as peristalsis and segmentation. These motility patterns ensure continuous mixing and contact between the chyme and the small intestine walls.

Nutritions is absorbed through the small intestine walls, which are composed of hundreds of microvilli. The microvilli host digestive enzymes and increase surface area to facilitate nutrients absorption. The digestion process in the small intestine occurs for 3-4 hours under a neutral pH environment. Nutrients released from small interstine will become bioaccesible to be transported through blood system (Kristensen *et al.*, 2021).

Some engineering solutions to study food digestion in the small intestine include the application of saline solution with digestive enzymes; the utilisation of mechanical tools to imitate paristaltis and segmentations movement; and the use of cell models semi-parmeable membrane to simulate nutrients absorption. The assessment of released nutrients can be done by spectrophotometer, chromatography analysis and nutrient measurement techniques from the chyme sample. In-silico modelling is also used to study digestion processes in the small intestine (Muttakin, Moxon and Gouseti, 2019,).

The chyme from the small intestine is passed to the large intestine or colon as a final food digestion process. The colon mucosa wall is covered by bacteria undertaking a fermentation of residual food components. The digesta passing throughout the colon with peristaltic and segmentation force at the same time released water to the colon wall and gas production of the fermentation (Guyton and Hall, 2015). The engineering

solutions to study food digestion process in colon phase include the use of prebiotics and microbiota profiling. Undigested component including resistant starches and non-starch polysaccharides are fermented into short chain fatty acid as key ingredient for the health. Some research suggested that controlling the microflora has a good influence on health (J. E. Norton *et al.*, 2014).

The gastrointestinal tract is a complex system that contains numerous control points, ranging from the degree of mastication to the rate of transit. Thus, comprehensive and complete food engineering systems are crucial for understanding how food is metabolized in the human body during digestion.

2.2. In-vitro Digestion Models

Human digestion system is an extremely complex system that consist of huge number of biological interactions, such as cells, membrans, genes, organics and inorganics compounds. This complexity makes it difficult to distinguish the associations between singular parts and to investigate how the processes happens. *In-vitro* digestion model is laboratory experiment conducted outside digestive tract. It's simplifying those complex system so the researcher can give attention to the specific part of components.

Reasons to seek in-vitro models may vary from academic curiosity, to convenience or to specific research needs. For example, in-vivo tests are typically laborious, expensive, time-consuming and often ethically compromised; while they provide valuable information about the digestion of a meal test, they rarely contribute to mechanistic detailed understanding of specific digestive processes; and gathered

data are often difficult to interpret in a predictive manner. For these and other reasons, in-vitro and or in-silico approaches to digestion are often being sought.

Depending on the specific experimental design, in-vitro models may mimic all stages of digestion or focus on a specific site (e.g. gastric); they may be mono-compartmental or multi-compartmental, in which the different stages of digestion are simulated in the different compartments; they may further analyse a small amount of material (e.g. in the order of mg) or a large, bite-size substance; or they may be static.

To gain accurate results as close to the its natural process inside the body, a complete in-vitro model would simulate all digestive processes as well as being a simple to utilise and fast process. It would likewise be adaptable in its capacities to take into consideration any affectability investigation or varieties in the stomach related conditions wanted to be contemplated. It would likewise be reasonable to work.

In-vitro models usually designed specifically to suit the researcher's main goal. Numerous models have been developed, starting from oral phase, gastric phase small and large intestine phase to static and dynamic models. There are also a number of physiological aspects and environment of digestion might be consolidated in *in-vitro* equipments. Those include the temperature, pH and pH inclinations, the protein sorts, fixations and flow rates, addition of digestive liquids, for example, water, electrolytes or mucins, the volumes and flow rates of the foods, retention times, the motility of and mechanical activities applied by the stomach related tract, the dispersion and mass transfer, or the absorption of a various nutritions.

In-vitro digestion models had been widely classified into two different types, static and dynamic depending on whether temporal modifications of digestion are integrated or not. This might also refer to mechanical movement, gut wall

contractions, fluid circulation, within, and out of the digestive element this is simulated, and many others. Static gastrointestinal models offer simple, fast and flexible applications. While dynamic models offer more comprehensive systems with integration of physical movement, shape and physiological condition (Muttakin, Moxon and Gouseti, 2019).

2.2. Static in-vitro digestion models

Static models usually consist of typical each step of digestion phases with simulated digestive liquid and controlled mixing process. Each phase might be processed in the same tube or transferred to the series of tubes. The mixing devices such as rotator, magnetic stirrers and shaking incubators used to homogenize the food sample. The amount of sample also vary from micrograms to grams.

Various techniques on static *in-vitro* models are available to be reproduced by scientists. Starch hydrolysis measurement is one of the simple static *in-vitro* digestion assay. This method is useful for glycaemix index quantification with starch digestible classification defining base on hydrolysation time. Rapid digestible starch (RDS) represents the percentage of digested starch in 20 minutes. Slow digestible starch (SDS) term is for digested starch after 20 minutes up to 120 minutes. Then resistant starch (RS) is undigested starch after 120 minutes (Goni et al. 1997; Englyst et al. 1996; Englyst et al. 2003). The enzymatic reaction by amylase and amyloglucosidase enzymes were done to quantify the released glucose from food samples, such as rice (Hsu et al. (2015); Dhital *et al.* (2015); Van Hung, Chau and Phi (2016); Hung *et al.* (2016); Chen *et al*. (2017), bread (Ronda *et al.*, 2012) and oat (Brahma, Weier and Rose, 2016). Buffer solutions, for example phosphate buffer saline, sodium acetate buffer

(Van Hung *et al*. (2016); Chen *et al.* (2017)), may be used replacing human or animal digestive fluid.

The estimation of RDS, SDS and RS were calculated based on the digestion time with below formulas (Chen *et al.*, 2017):

$$RDS\ (\%) = \left[\frac{G_{20}-FG}{TS}\right] \times 0.9 \times 100 \quad (1)$$

$$SDS\ (\%) = \left[\frac{G_{120}-G_{20}}{TS}\right] \times 0.9 \times 100 \quad (2)$$

$$RS\ (\%) = \left[\frac{TG-FG}{TS}\right] \times 0.9 \times 100 - (RDS + SDS) \quad (3)$$

where G_{20} represent the glucose content after 20 minutes, G_{120} is for 120 minutes digestion process. Then, FG is a results measurement of free glucose in supernatant of distilled water extracted starch and TG is the total glucose after digestion process.

More complex techniques in static digestion assay comprise each step of the digestion process. *In-vitro* digestion experiment conducted in the experimental tube, such as glass flask, conical flask or jacketed laboratory beaker. Simulated digestive fluids containing electrolyte solutions, enzyme and water are explicitly used during each phase. The liquids are simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The experimental environment such as temperature, pH, sample particle size, sample matrices were used based on research purpose.

Various protocols have been developed by scientists in term of mimicking digestion process statically. COST action Infogest, a group of food scientists working

in Europe have published a standardised static *in-vitro* digestion protocols (Minekus *et al.*, 2014). This INFOGEST protocols might be an excellent option to be replicated as it was an international consensus.

INFOGEST protocols describe step-by-step protocols of static in-vitro digestion from oral, gastric to intestinal phase. The summary of protocol is shown in Figure 1. Mastication process in the oral phase was mimicked using a meat mincer with additional SSF and salivary amylase enzyme. Further process in gastric phase, SGF being used on 1:1 composition with bolus weight and 2000 U/ml of pepsin as active compound to digest food in gastric phase tube. Finally, in intestinal phase, chyme should be mixed with 1:1 of SIF and intestinal enzyme, which are individual enxymes or enzyme extract. Samples collections is done depend on the research purpose, it is suggested that each sample is processed using individual tube. Avoiding taking sample from same tube will give more valid data and easier sampling process.

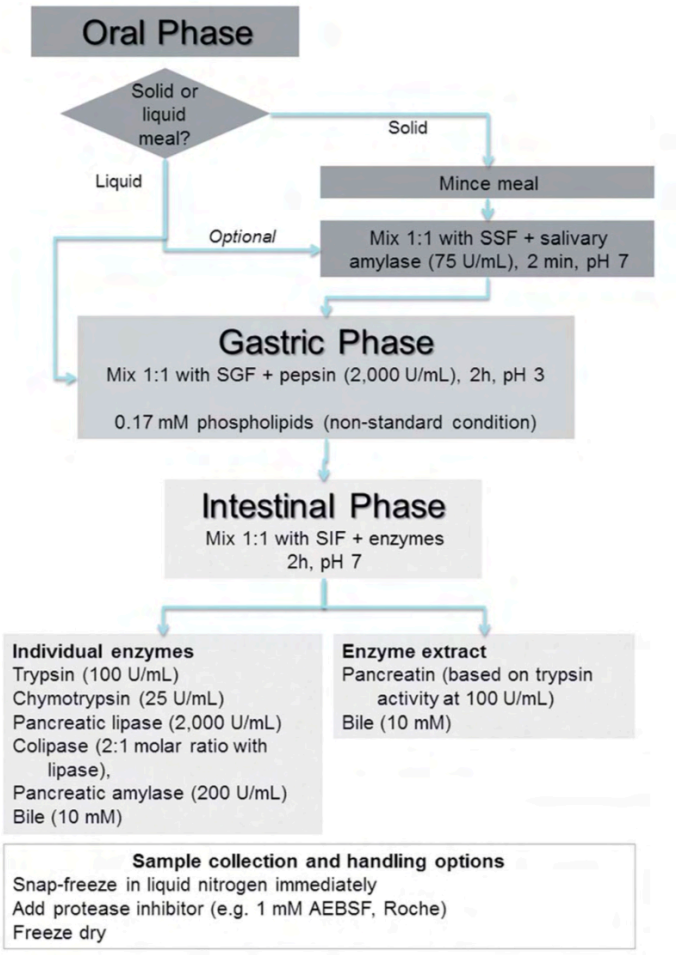


Figure 2. Diagram of simulated digestion process on each phase with the amount of electrolyte solution, enzyme and other substances (Minekus *et al.*, 2014).

Comprehensive works have been done by INFOGEST scientists to test the consistency of standardised static in-vitro methods on the protein digestion process. This harmonised study was a collaboration of three laboratories, exhibiting the INFOGEST method (Minekus *et al.*, 2014) shown a consistency which can be a good reference of in-vitro research for future replication. However, a direct comparison with in-vivo data would be an essential way to validate the methods (Egger *et al.*, 2016). Another in-vitro static intestinal work used to evaluate lipid digestion has been developed and reproduced as well (Salvia-Trujillo *et al.*, 2013; Qin *et al.*, 2016; Mun and McClements, 2017; Ban *et al.*, 2018).

2.3. Dynamic in-vitro digestion models

Similar with static models, dynamic models may reproduce at least one section of the digestive system. In advance, dynamic models offer the capability of including component of the near-realistic and physiological condition, for example, mechanical forces or peristaltic movements, fluid flow, temperature and pH of surrounding environment, type and concentration of enzymes, and even a similar organel shapes. Various dynamic gastrointestinal models have been developed by the researcher. Some of them have been published as seen in Table 1. It ought to be noted that the list of Table 1 is not comprehensive, yet rather characteristic.

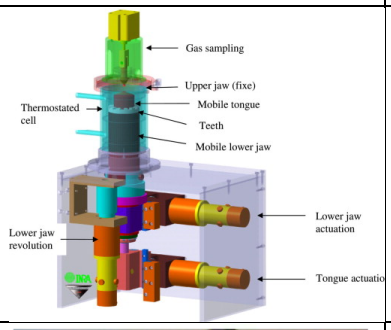
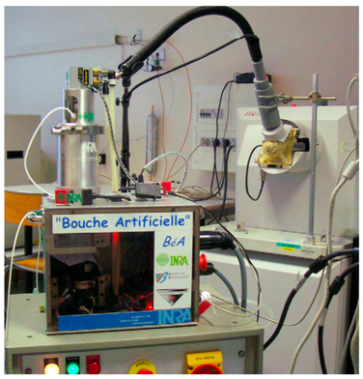
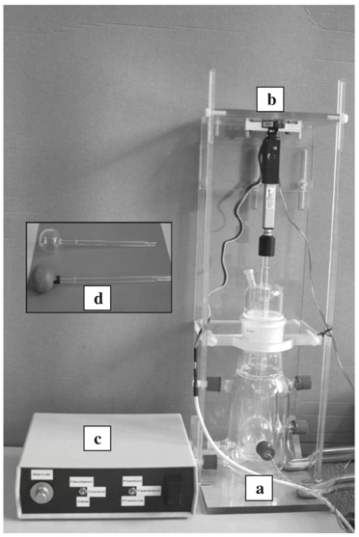
In-vitro models study bioaccessibility. Foods typically need to be broken down mechanically and hydrolysed by enzymes before absorbable compounds are released (Marze, 2017). The oral phase in digestion process is a major step influencing nutritional intake and digestibility of food compounds as results of particle breakdown from the mastication process, bolus formation, saliva secretion and activity of salivary enzyme. In terms of preparing food for bolus formation, *in-vitro* oral digestion phase have been mimicked using blender (Bordoloi, Singh and Kaur, 2012; Dhital *et al.*, 2015; An *et al.*, 2016; Tamura *et al.*, 2017), meat mincer (Bornhorst and Singh, 2013), and sophisticated mouth models (Salles *et al.*, 2007; Mielle *et al.*, 2010; Benjamin *et al.*, 2012; Panouillé *et al.*, 2014).

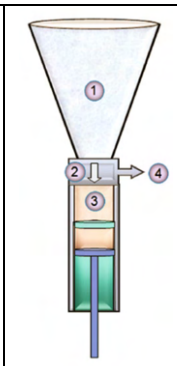
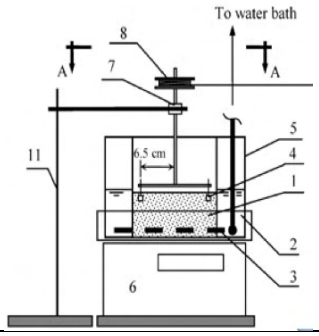
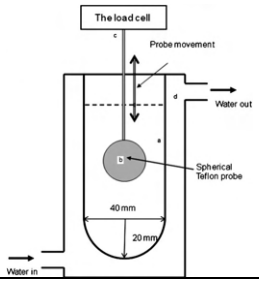
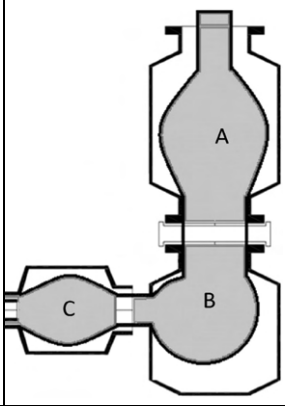
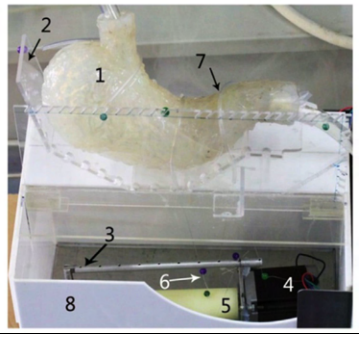
Some researchers have developed the dynamic gastric models, for examples the Dynamic Gastric Model/DGM (Siegel *et al.*, (1988); Kong and Singh, (2008, 2009)), Dynamic Duodenum/DDuo (Tharakan *et al.*, 2010; Gouseti *et al.*, 2014) as presented

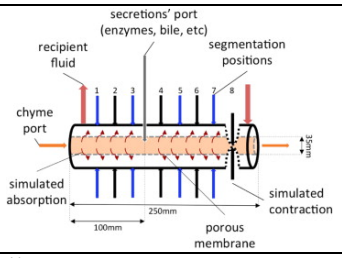
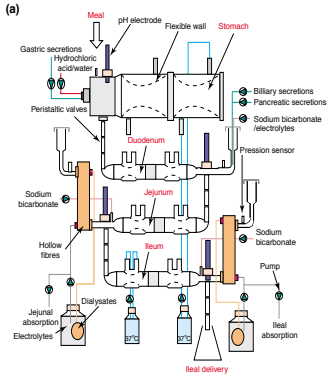
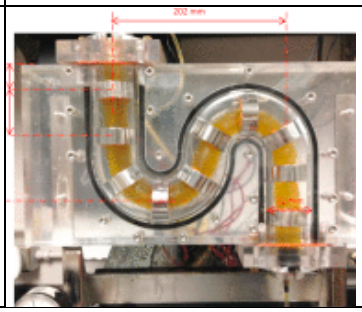
in Tabel 1. Human Gastric Simulator (HGS), a vessel siting on a turntable mimics gastric physical forces utilized on food digesta through the rough movement of plastic beads (F Kong and Singh, 2008). Chen *et al.* (2011) was replicating gastric fluid flow by the action of a vertically moving spherical bead positioned in a jacketed tube.

The latest human gastric models named TIMagc and RD-IV-HSM have been developed by groups of researcher. TIMagc model was claimed has similar accurate dynamic condition as human stomach (Bellmann *et al.*, 2016). RD-IV-HSM, introduced as a the closest realistic of human gastric model providing realistic gastric morphology as well as mimicking the physical movement (Chen *et al.*, 2016).

Table 1. In-vitro dynamic gastrointestinal models

Model	Schematic	Mechanism to produce mechanical action	References
In-vitro mouth model		Mimics mastication process similar to mouth movement with artificial teeth, jaws and tongue.	(Salles *et al.*, 2007)
Chewing machine		Replicates human mouth mechanism; connected on-line with API-MS to monitor flavour release.	(Mielle *et al.*, 2010)
Artificial tongue		Mimics the pressure pattern of human tongue; has computer controlled artificial tongue; connected on-line to the volatile organic compounds measurement device	(Benjamin *et al.*, 2012)

Dynamic Gastric Model (DGM)		Complex wall motion was used to mimic gastric wall contractions; Simulation of gastric motility was by squeezing of the conical shaped vessel	(Wickham, M.S.J.; Faulks, 2013) Mercuri et al., 2008; Lo Curto et al., 2011)
Human Gastric Simulator (HGS)		The model mimics mechanical forces utilized in stomach by abrasion of article-particle	(F Kong and Singh, 2008)
Gastric Model		This model used compression action between cylindrical wll and spherical probe to mimic fluid flow.	(Chen *et al.*, 2011)
TIMagc (advanced gastric model, dynamic and computer-controlled)		Mimics the motility and segmentation peristaltic forces of the stomach, in- cluding the housekeeperwave	(Bellmann *et al.*, 2016)
RD-IV-HSM (Human gastric model)		Providing realistic gastric morphology as well as mimicking the physical movement	(Chen *et al.*, 2016)

Dynamic Duodenal Model (DDuo)		Mecanichal action by squeezing the wall of tube represented segmentation and peristaltic movement.	(Tharakan et al., 2010; Gouseti et al., 2014)
TIM1 (gastric and small intestinal model), similar apparatus exists for large intestinal simulation		Mechanical force by squeezing flexible tube wall	(Minekus et al., 1999; Marteau et al., 1997; Blanquet et al., 2001; Krul et al., 2000)
Human Duodenum Model (HDM)		Simulate segmentation movement and has sigmoidal shape.	(Wright *et al.*, 2016)

A combination of digestion phases also introduced as *in-vitro* models. TNO's Intestinal Model (TIM1) used different compartment to replicate gastric, duodenal, jejunal, and ileal (Minekus *et al.*, 1995; Marteau *et al.*, 1997; Krul *et al.*, 2000; Blanquet *et al.*, 2001). Extension works on the second model (TIM2) included the large intestinal phase (Minekus *et al.*, 1999). The models in dynamic were able to replicate peristaltic contractions by a squeezing the walls of a cylindrical tube and controlling secretion, pH and temperature.

The human duodenum model (HDM), published in 2016 shows a more sophisticated tool to study food component breakdown and absorption in the intestinal track. HDM comprises segmentation function and sigmoidal shape (Wright *et al.*, 2016).

Overall, mimicking the food digestion process is challenging because of the complex system of the human digestive system and the food matrices. An extensive assortment of in-vitro food digestion models have been introduced and gain popularity more over the last decades. They may consider at least one digestive process in a simple or very sophisticated way, and they are very considered as application specific. Static models are easy to operate and relatively fast, compared to the laborious and time consuming dynamic models. However, dynamic models represent more accurately the physiological processes occurring in-vivo. When selecting the appropriate in-vitro model we ought to comprehend the impediments and points of interest of every choice and consider the model that tends to our inquiry.

2.4. Glycaemic Index (GI)

The glycaemic index is an indicator on a scale from 0 to 100 of a potential carbohydrate food increasing blood glucose level. The indexing mainly comparing glycaemic response of test food with reference food (for example; glucose or white bread) consumed by same subject. The most important determinant of glycaemic response are the rate of food digestion together with insulin response (Jenkins *et al.*, 2002). Thus evaluating digestibility of starchy staple food are still a proposed topic by some researchers.

Several studies on food digestibility and glycaemic index assay have been published. *In-vitro* enzymatic digestion process was a starting point to gain the hydrolysis index (HI) used for glycaemic index calculation. The equation to determine estimated glycaemic index (eGI) as follow.

$$C = C^{\infty}(1 - e^{-kt}) \quad (4)$$

where C is the hydrolysis degree at time points; C^{∞} stands for the maximum hydrolysis degree; and k as the kinetic constant. When the area under the hydrolysis curve of each sample is divided by area under a reference (fresh white bread), the result is known as Hydrolysis index (HI). The eqution of estimated glycemic index is as follows. (Wolter *et al.*, 2013).

$$eGI = 39.71 + 0.549HI \quad (5)$$

2.5. Dietary Fibre and Glycaemic Index

Dietary fibre has been known as a healthy ingredient, due to its prebiotic function. It is also claimed it can reduce the risk of developing gut-related disorders including cardiovascular diseases and diabetes. Hydrocolloids (particularly dietary fibre) present some benefical use to formulate healthier meal through controlling gastric emptying given effect to satiety and obesity, lowering glycaemic response and level of plasma cholesterol and also creating good environment for carbohydrate fermentation in large intestine (Gidley, 2013a).

Hydrocolloids change bioaccesibility and nutrient mass transfer of digested food. The simplified in-vitro intestinal models developed by Gouseti et al. (2014) could mimic chime flowing as well as mixing process in the small intestines. It has been proven that Guar gum, CMC, and pectin reduce the release rate of glucose by 30% compared to distilled water in-vitro.

Mackie, Bajka, & Rigby (2016) observed that the dietary fibre had a scope of usefulness in the early parts of GI tract. They concluded that the addition of fibre could change gastric emptying rate which affects the full stomach feeling and satiation. These mechanisms promoted by viscosity changing, nutrient release and sensing in the duodenum.

Foschia, Peressini, Sensidoni, Brennan, & Brennan (2015) studied in-vitro starch digestion of pasta with the addition of dietary fibre. They concluded that the predicted glycaemic response reduced by the addition of dietary fibre (glucagel, inulin, psyllium and oat) to substitute 15% of durum wheat semolina. A study by Brennan, Derbyshire, Tiwari, & Brennan (2012) showed that potential glycaemic response was significantly lowered by the addition of mushroom coproduct material to extruded snack products.

2.6. Paracetamol as a solution model

Paracetamol has been used as a solution model due to its solubility in water (Granberg and Rasmuson, 1999) and simple detection method using spectrophotometric (Shrestha and Pradhananga (2009), Sawant *et al.* (2012) and Hoang *et al.* (2014)). Behera *et al.* (2012) developed and validated a assay of Paracetamol's tablet formulation in a ultraviolet visible-region. The paracetamol shows maximal absorbance at wave length 243. This method is simple, accurate and reproducible.

Wickham *et al.* (2012) reviewed the design, operation and application of dynamic gastric models, which include the use of paracetamol as a measured substance. With regard to predicting human *in-vivo* performance, Vardakou *et al.*, (2011) evaluated the release of paracetamol as a rapidly dissolving model drug in conventional and novel gastric compartments.

2.7. Hydrocolloids and its effect in digestion

Researchers are increasingly focused on ways to reduce chronic cardiometabolic disease risks by reducing the blood glucose response to carbohydrate-rich meals. The investigations included an attempt to convert RDS to SDS with the addition of viscous (such as guar gum, gum arabic) or gel-forming (alginates, gellan gum, pectins) hydrocolloids under gastrointestinal circumstances (Boers, Hoorn and Mela, 2016). Starch, modified starch, xanthan, galactomannans such as guar gum and locust bean gum (LBG), gum Arabic or acacia gum, gum karaya, gum tragacanth, and carboxymethyl cellulose have all been utilized as thickening agents in a variety of food systems. Alginate, pectin, carrageenan, gellan, gelatin, agar, methylcellulose, and hydroxypropylmethylcellulose are all prominent gums that are used in food as gelling agents (Saha and Bhattacharya, 2010).

Hydrocolloids have significant effect to impeded simulated glucose accessibility (Gouseti et al., 2014). Chen et al. (2017) found that rice starch digestibility changed by the addition of pullulan (PUL), persuading significant growth of SDS and RS contents. It was speculated that the inhibitory effect of pullulan on gelatinization process and its coating effect to be in charge of diminishing digestibility of rice starch.

The possibility of incorporate hydrocolloids into food and their effect on starch digestibility are presented in the following studies. The RS4-coated rice showed lower starch digestibility, a decreased glucose response and a slower rate of blood glucose. It was discovered that a 0.1% mixture of locust bean gum (LBG) and agar was the optimal coating solution for preparing RS4-coated rice (Choi et al., 2010). Additional inulin induced a reduction of rapidly available glucose (RAG) and starch digestion rate index (SDRI) of wheat bread (Ronda *et al.*, 2012).

According to the correlation findings, pectin's degree of esterification affects in vitro starch digestion and pancreatic amylase activity. Polygalacturonic acid (PGA) increased the resistant starch production by 20 per cent compared to the control group, which could be advantageous to human health (Bai *et al.*, 2021). Pectin inhibits in vitro enzymatic starch digestion; first-order kinetics are observed with and without pectin, with the rate coefficient for starch/pectin being much lower than that for starch alone (Bai *et al.*, 2017).

Incorporating arabic gum (0.5–1.5 percent) lowered the glucose release curve and lowered the pGI of Segoami noodles considerably. The higher the arabic gum content, the greater the cooking loss; nevertheless, the hardness and cohesiveness were decreased. Since arabic gum reduced in vitro starch digestibility and improved Segoami noodles' cooking qualities, it was shown to be the most effective amount to use in the final product (Bae *et al.*, 2019).

2.8. Gellan gum

Gellan gum is a hydrocolloid origin manufactured by the fermentation activities of bacteria *Sphingomonas elodea* (CP Kelco US, 2007). Gellan gum is commercially available as a food additive ingredient for the food and beverage industry, mainly have used as a thickening agent, controlling the viscosity and structuring the product. It is offered in two forms: high acyl (HAG) and low acyl (LAG). These two forms of gellan gum are characterized by the method used to recover the polysaccharide. The form of substituted native, or high acyl (HAG), is recovered directly from the broth via alcohol precipitation. Alternatively, before alcohol precipitation, alkali treatment leads to deacylation and the unsubstituted, low acyl

(LAG) form (Sworn, 2009). Figure 3 shows the chemical structure of high acyl and low acyl gellan gum.

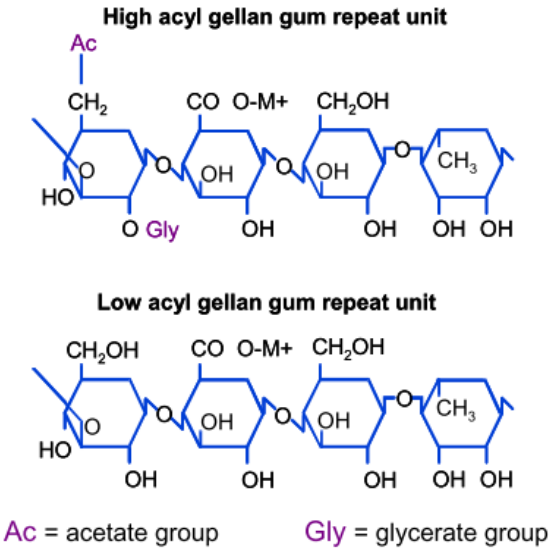


Figure 3. Gellan gum structure of high acyl and low acyl (CP Kelco US, 2007)

Gellan gum has a linear chain structure composed of repeating glucose, rhamnose, and glucuronic acid units. Two acyl substituents – acetate and glycerate – are present in their native or high acyl form. Both substituents are attached to the same glucose residue, and on average, one glycerate and one acetate are present in every two repeats. The acyl groups are entirely eliminated in low acyl gellan gum. The acyl groups have a significant effect on the gel properties. A high acyl content results in soft, elastic, non-brittle gels, whereas a low acyl content results in rigid, inelastic, brittle gels (CP Kelco US, 2007).

Study found that low acyl gellan gum (LAG) has self-structuring-gel ability in low pH environment (Norton, Cox and Spyropoulos, 2011). The addition of LAG and HAG gellan gums modified the rheology and textural properties of rice starch gel (Fang *et al.*, 2018). LAG could be a potential ingredient to delaying gastric emptying time and lowering the digestion rate of foods. These findings were leading a further investigation on the digestion of food with additional LAG.

2.9. Rice

Rice as the highly consumed main food plays important rule to maintain health of the world population. Rice kernel is composed in various layers. Following the de-husking process, the remain of the rice portion is called brown rice. It consists of bran as an outer layer and the internal endosperm as inside layer and embryo in the core. Further polishing process which remove pericarp and aleurone layer produces white rice (Wang *et al.*, 2015).

Compared to white rice, brown rice are rich in nutrients and bioactive compound has higher content of protein, soluble and insoluble dietary fiber, (Bornhorst *et al.*, 2013). Present of various layer on brown rice effects its digestibility. During gastric digestion, brown rice has a greater particles size distribution than white rice (Bornhorst, Kostlan and Singh, 2013). Furthermore these results were giving slower mass transfer rate of nutrient absorption in intestine (Tharakan *et al.*, 2010).

The health effect of rice seems to be primarily attributed to its glycaemic load in the rice consuming population. Atkinson et al., (2008) presented a systematic GI value tabulation which exhibits rice as product with GI value varied between 24 and 160 (white bread as reference).

The rice GI depends on amylose content categorised as waxy (0-2 percent), very low amylose (5-12%), low amylose (12-2%), medium amylose (20-25%), and high amylose (25-33%). The rate of starch digestion is affected by amylose and amylopectin ratio which was used to predict the responses of blood glucose and insulin to rice consumption (Kaur, Ranawana and Henry, 2016).

The literature indicates that rice with high amylose have a lower GI number than high amylopectin variants. However, glycemic response, starch digestion rates

and glycaemia are often influenced by other factors as well as amylose content (Kaur, et al 2016).

Rice processing affects the GI value. Processes were changing structure formation, and facilitating gelatinization increases the GI of rice. Meanwhile, GI reduced by the promotion of RS formation and complexes of amylose/amylopectin. Moreover, the chemical composition plays a vital role in the rice starch digestibility. Parboiling can decrease rice GI. This GI value depended on the parboiling process used and the cooking condition (Dhital et al., 2015). In addition, extruded rice products found has a lower GI value than rice grain. However, the value is dependant on the type of rice, drying process and the cooking methods (Srikaeo and Arranz-Martínez, 2015).

While rice variety and cooking process influence rice digestibility, another aspect that should be considered to engineer healthier rice product is customer acceptance. Experimental studies have shown that in milled rice, the aylose content was positively associated with the overall sensory taste. Then, the crude protein content was found negatively correlated with overall palatability (Kesarwani *et al.*, 2015). As brown rice or less-milled rice has more protein content, its general consumer acceptance might be lower than white rice. The increase of milling degree increases L* colour parameter, which is showing a brighter colour, gives a positive correlation in eating quality (Zhong *et al.*, 2014).

The gap between creating healthier rice product and its sensory acceptance opens a broader area to be explored. One of the methods that can be used in lowering white rice digestibility was using additional hydrocolloid in the cooking process. For example, in the presence of Pullulan, rice starch has a slower digestibility rate compared to a rice control (Chen *et al.*, 2017). A study of rice starch modification with

addition carrageenan and gellan has shown an improving in adhesiveness and hardness of that rice starch. This finding will be useful for creating food using rice flour Huang *et al.*, (2007).

The finding on slower rice digestibility with addition hydrocolloid have potential for its application on food matrix for example cooked rice. Thus, some studies need to be done to develop new way to prepare rice that will have a lower GI. These indeed require a deep understanding of the digestion mechanism for rice.

CHAPTER 3

Developing Methods

As part of the aim on develop new methods in investigating digestion and release of macronutrients relevant to glycaemic index, some initial studies have been conducted. The digestion aspects that need to be investigated are the effect of dilution to the nutrient bioaccesibility, effect of gut motility on nutrient bioaccesibility and the mechanism that lead to bolus breakdown and release of nutrient from the food matrix.

3.1. Gastric Models: Effect of Dilution

There is a wealth of knowledge on absorption of relatively simple molecules, e.g. drugs (Ehrhardt and Kim, 2008). We will build on this knowledge to understand the effect of dilution on the bioavailability of molecules. In this case new *in-vitro* experimental methods were built for studying the absorption of a soluble drug compound after oral administration. In this preliminary works, we used Paracetamol as a model solution. Paracetamol has high solubility in water with values of 7.21 g/kg at 0 °C, 8.21 g/kg at 5 °C, 9.44 g/kg at 10 °C, 10.97 g/kg at 15 °C), 12.78 g/kg at 20 °C and more than 14 mg/ml at 20 °C. within the concentrations (Granberg and Rasmuson, 1999) which will be achieved during the experiments. Moreover, paracetamol can be detected directly using UV Vis Spectrophotometer without addition sample treatments.

Most *in-vitro* models do not utilise a compartment to mimic the absorption of

the drug from the intestinal compartment and as such over estimate the effect of precipitation with the intestinal compartment. To avoid this effect an *in-vitro* method will be proposed where a permeable membrane is used between a source and sink compartment, but in the method proposed, the source compartment (representing the proximal small intestine) will be submerged in to the recipient compartment (representing the blood supply). The main aims of the study were to examine if absorption profiles can be achieved in-vitro which are similar to what will be expected in vivo.

3.1.1. Experimental Procedures

The experimental work consisted of two stages, a setup involving a permeable membrane to measure the passive transfer across the dialysis tubing, and a compartment setup which had a transfer from a gastric to duodenal compartment, and a measurement of the concentration of dissolved drug in the duodenal compartment. The experiments consisted of two different set ups, the first containing two compartments, and the second containing 3 compartments. Figure 2 shows the schematic of the 2 compartment method. The experiment consists of a cylindrical metal frame with a diameter of 75 mm, this is covered with a permeable membrane, the membrane is made up from 3.5 kDa dialysis tubing (snakeskin dialysis tubing, Thermo Scientific), with the bottom of the cylinder fixed with non permeable material, giving a surface area for absorption of πDh, where D is the diameter of the frame, and h is the height of the liquid in the compartment. Inner compartment was filled with a solution of the drug, it was then submerged into a jacketed vessel, containing distilled water at 37 °C.

3.1.1.1. 2-Compartmental Experimental Set up

The two compartmental set-up has the submerged permeable membrane frame in the jacketed vessel of distilled water as outlined in Figure 4. Compartment 1 was mixed with a overhead stirrer (LS Overhead Stirred Velp Scientifica) at 300 rpm, and a magnetic stirrer was used to ensure the content of the second compartment is mixed. The drug solution was in compartment 1, and the rate of mass transfer was quantified by taking samples from the second compartment, every 5 minutes over a total 60 minutes, and the absorbance measured with a UV spectrometer at 300 nm wavelength, to quantify the drug concentration.

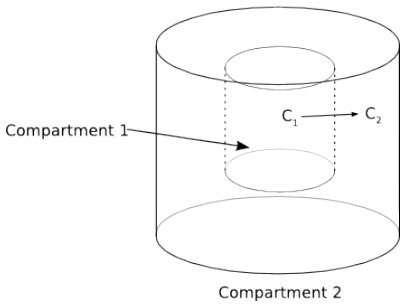


Figure 4. The 2-compartment experiment set-up.

3.1.1.2. 3-Compartmental Experimental Set up

The second set of experiments had the same set up as the 2-compartmental system, but there was a 3rd compartment with a circulation to-and-from the 2nd compartment at a volumetric flow rate, Q, controlled by a peristaltic pump. In this case, samples were taken from both compartment 2 and 3, at the 5 minute intervals over a 60 minute period. The 3-compartment set up shown in Figure 5.

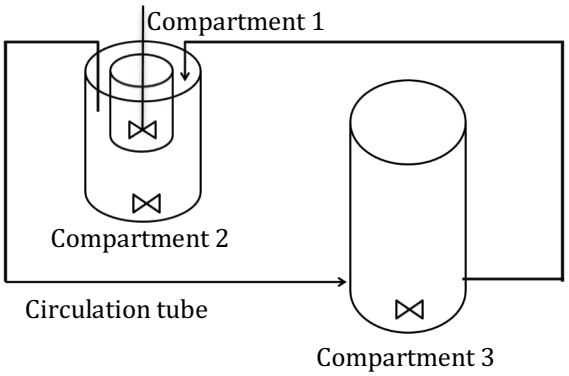


Figure 5. The 3-compartment experiment set-up.

3.1.2. Experimental Results

Experiments conducted with different volumes in the compartment 1 (100 ml, 150 ml, and 200 ml), these experiments were conducted with a paracetamol solution of concentration 2 mg/ml. An experiment was also conducted with a paracetamol solution of 1 mg/ml concentration solution in with 150 ml volume. The results are shown on Figure 6.

The increase in absorption with increase in volume for the 2mg/ml solution is likely down to the rise in available absorption surface area. Due to the fixed diameter of the tubing, increasing the volume also increases the surface area. This is also expected *in-vivo*, a greater volume of solution entering the proximal small intestine would have greater contact with the intestinal wall. It is noted that the available surface area is much smaller than would present in vivo, due to the presence of villi and microvilli etc.

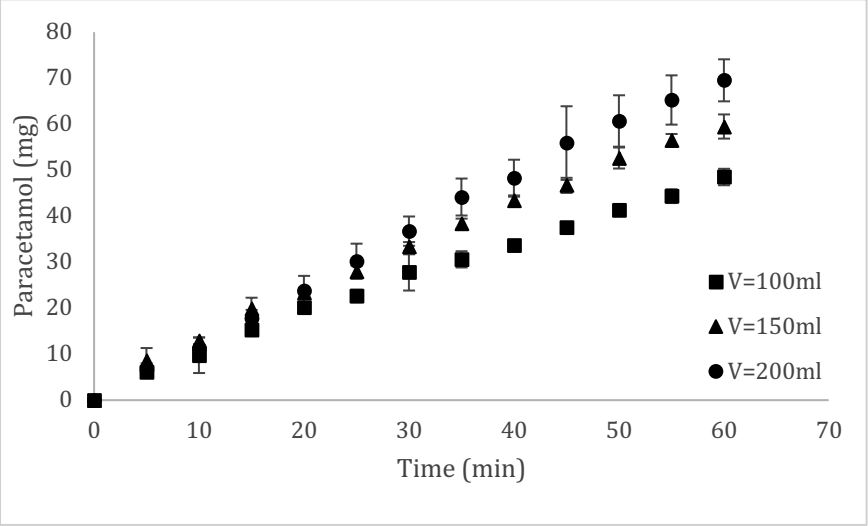


Figure 6. The mass of paracetamol (mg) transferred into compartment 2 for the different experimental conditions

The 3-compartment system behaved the same as the two-compartment system but there was a flow between the second and third compartments. This was to mimic the distribution of the drug in the blood supplying the GI tract (compartment 2) and the blood supplied to the rest of the body (compartment 3). The graph of paracetamol mass versus mixing time shows in the Figure 7.

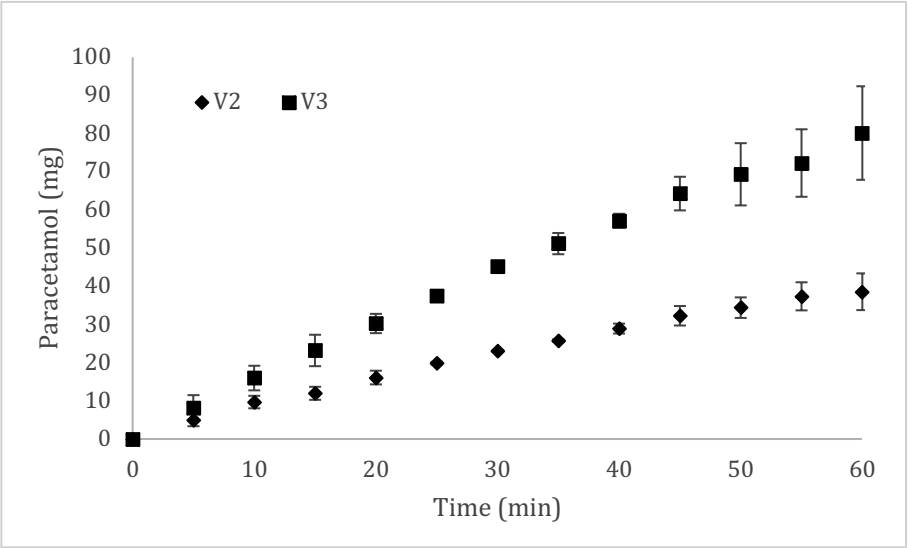


Figure 7. The mass of paracetamol within compartment 2 and 3

Dilution plays important rule in nutrient absorption. The study showed that the increasing dilution correlated positively with the amount of absorbed substance. This might be due to the higher chance contact rate of drug particle with the permeable membrane. The results should be applicable when food materials are used as the sample.

According to this initial experiment, it is possible to use stirred tanks to characterise some of the nutrient absorption phenomena. Furthermore, to mimic the nutrient absorption in the small intestine affected by peristaltic movement, a further study is needed to characterise forces as they will affect the mass transfer.

3.2. Trial of dynamic duodenum model equipment

An *in-vitro* dynamic duodenal model food system was developed by food digestion research group led by Prof. Serafim Bakalis at the University of Birmingham, University of Nottingham then Copenhagen University. The model can delineate the effect of intestinal motility in the mass transfer of food nutrients. The model mimics segmentation and peristaltic action to persuade mixing prosesses as they happen in small intestine.

The early version of small intestine model was unable to stimulate peristaltic and segmentation forces to study the mixing and mass transfer as the movement was designed using a pair of cuffs. Details of an early version small intestine model has been described in elsewhere (Tharakan, 2009; Tharakan *et al.*, 2010; Fonseca, 2012) and a schematic drawing of the model as Figure 8. The latest version of dynamic duodenal model (DDM) used in this experiment developed earlier as describe by Latty (2019) with modification in duodenal tube. Figure 9 shows the schematic diagram of DDM and the actual appearance of DDM as shown in Figure 10.

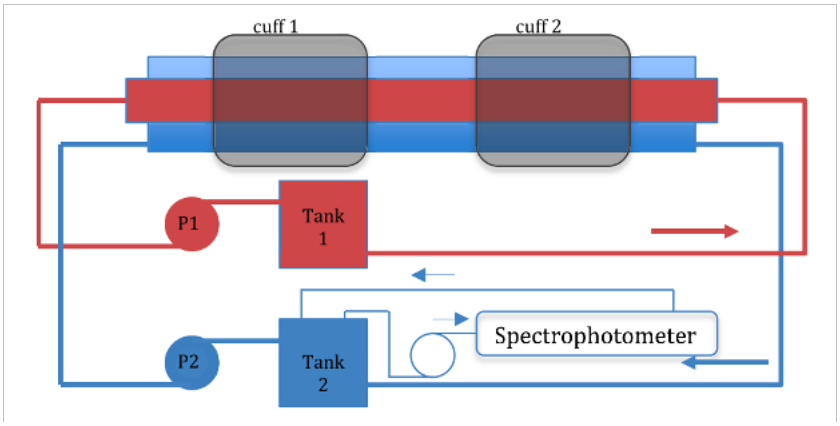


Figure 8. Schematic drawing of Small Intestine Model (SIM) as described by (Gouseti *et al.*, 2014)

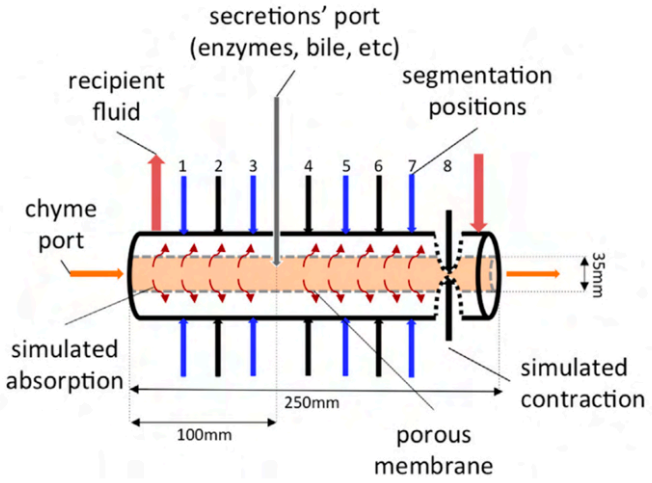


Figure 9. A schematic diagram of Dynamic Duodenal Model (DDM) as describe in Gouseti *et al.*, (2014)

Some simple experiments have been conducted to trial the dynamic duodenum model (DDM) equipment developed by University of Birmingham. DDM implements an automated and flexible design which allows to observe the digestion process effected by peristaltic and segmentation. The DDM uses a twin tube consisted of inner tube made from dialysis tube and the outer tube using silicon tube. Sample of digested chyme placed in the inner tube represented intestinal lumen. The area between inner tube and outer tube called as recipient area that is circulated distilled water connected to jacketed glass flask.

This experiment aimed to practise using DDM for simple digestion materials. The 1% glucose solution (from D-glucose powder, Sigma-Aldrich, US) in distilled water was used in this study compared with sample of additional 5% guar gum (Sigma-Aldrich, US) to the glucose solution. Distilled water was used as recipient from this part samples were measured for its glucose content.

Figure 10. The latest version of Dynamic Duodenal Model (DDM)

The procedure of this study was taken form Gouseti et al. (2014). Glucose solution (100 ml) was placed in the inner tube and outer tube filled with circulated distilled water from jacketed glass flask (temperature water bath 37°C). The segmentation occurred at 7 position every 10 s and looped after the last position. A series of samples was taken from recipient side every 5 minutes for 1 hours. Reducing sugar were measured using the dinitrosalicylic acid (DNS) assay.

Glucose content in the recipient side after being processed in the DDM showed in Figure 11. This simple trial experiment exhibits that additional 5% of guar gum to the glucose solution may reduce glucose absorption in the recipient side. Glucose

solution without guar gum gives significant higher released glucose. This finding has similar results with the study by (Gouseti *et al.*, 2014). The addition of dietary fibre decreases glucose absorption due to the increase of viscosity.

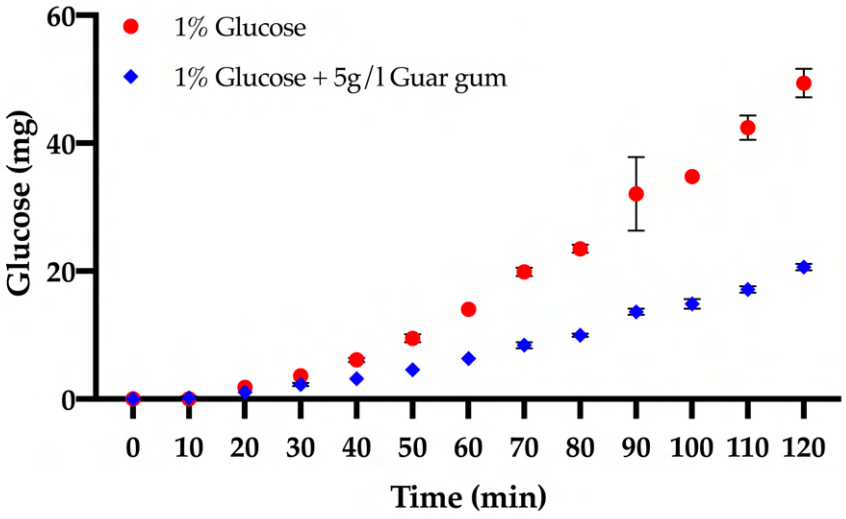


Figure 11. Glucose content in the recipient side (mg) after digested in DDM. Values are means of triplicates ± standard deviation.

This preliminary experiment showed that DDM could be a potential model to mimic the food digestion process in the small intestine. The presitaltic and segmentation movement can be easily managed based on the research purpose. Closed on both side of duodenal tube with permeable porous membrane was able to simulate chyme mixing and mass transfer from inside the permeable to outer side. This model acts to mimic nutrient absorption happening in the small intestine transfered to the blood system.

The challenging aspect to operate DDM was the making of duodenal tube and sampling technique. Duodenal tube should be assembled carefully to prevent leakage happening in the system. Taking the sample during in-vitro digestion in DDM is a tricky process, especially when a food matrix is used as study object. Open and re-close the duodenal tube should be done effectively, to make sure a proper sample is

taken and there are no leaking. A homogenous sample are needed to get accurate nutrient concentration for food digestion process measurement. Thus, a static in-vitro technique should be considered to use for it is simple and replicateable.

3.3. Static in-vitro digestion model

Static *in-vitro* digestion method offers simple protocol yet includes the mixing to mimic food digestion process in the human gut. This experiment using a standardised static *in-vitro* protocol, develop by The COST Infogest work group (Minekus *et al.*, 2014; Brodkorb *et al.*, 2019). The static model food digestion of Infogest is shown in Figure 12. The method of static digestion utilises constant ratios of food and digestive fluid and sustained pH in every digestion phase This method is convenient to use and all the parameters are calculated based on physiological data. Figure 13 shows the basic equipment to be used in this experiment, including chopper, tube rotator, centrifuge and UV-Vis spectrophotometer.

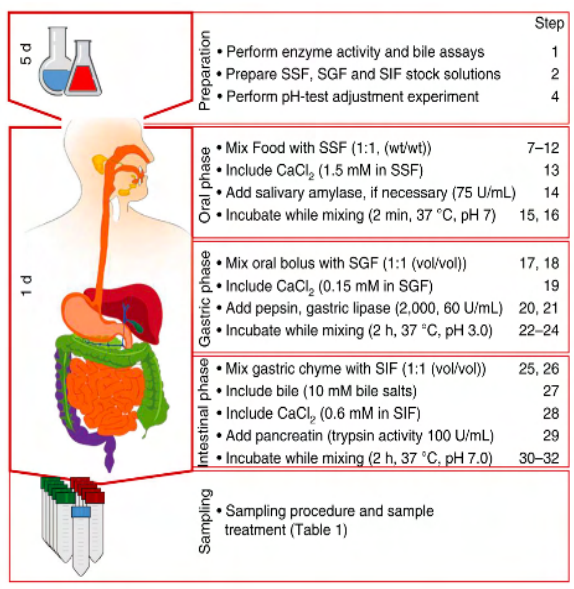


Figure 12. Flow diagram of the Infogest digestion methods (Brodkorb *et al.*, 2019)

Figure 13. Basic equipments for static *in-vitro* digestion methods

A preliminary work of the static *in-vitro* digestion model was conducted on ground jasmine rice with addition of hydrocolloids. The static digestion protocol was based on starch hydrolysis protocol for material selection by Quadram Institute, UK with some modifications. The amount of 131.58 mg (equivalent to 100 mg of starch) of ground jasmine rice (250 – 500 μm) with and without additional 1% of hydrocolloids material (carrageenan, pectin and gellan gum) were digested on 15 ml conical tube as follow procedures. The samples were cooked with 10 ml of distilled water for 20 minutes in the boiling water bath. Then the samples were cooled at room temperature for 10 minutes. The 0 minutes samples were taken as much as 200 μl then replaced with same amount of α-amylase solution to start the hydrolysis process. The tubes were attached on the rotator placed inside 37°C incubator. During the starch digestion process, 200 μl samples were taken on each time point (5, 15, 20, 30, 60, 90 minutes) to the stop solution (0.3M Na_2CO_3) contained 1.5 ml micro tube. The digested samples were then centrifuged (12500 rpm, 5 minutes) and diluted 20-fold. Sugar reduction contents were measured using PAHBAH method with 1 mM Maltose solution as standard. The starch hydrolysed (%) of jasmine rice samples as shown on Figure 12.

As shown in Figure 14, the starch of all the samples was hydrolysed around 30 minutes of incubation. After that time, the starches begin to reach plateau conditions, where most of the starches have been digested. During the first 30 minutes, jasmine rice powder with additional 1% gellan gum show the lowest hydrolysis rate while samples without additional hydrocolloid were the fastest being digested. These results confirmed the conclusion from published research that additional minor amount (0.3 and 0.7%) of commercial hydrocolloid could lowering digestibility of cooked rice (Chung, Liu and Lim, 2007).

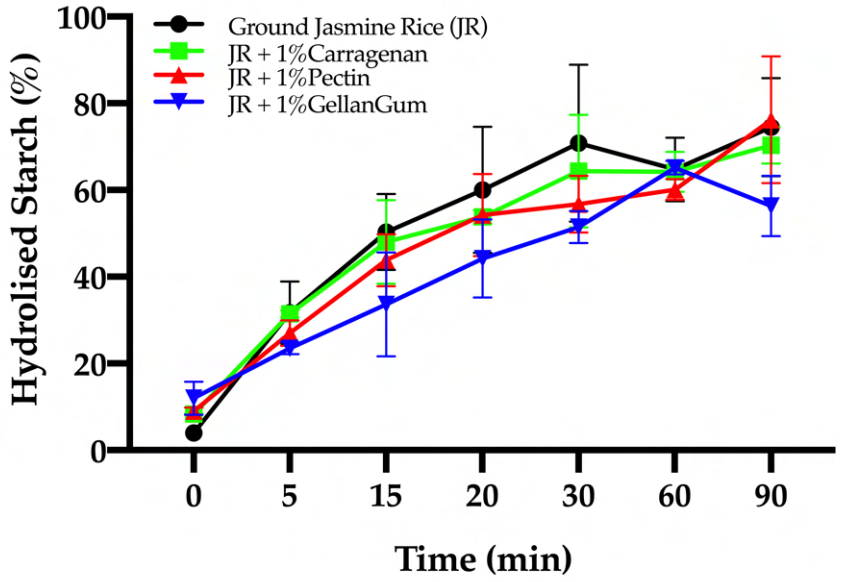


Figure 14. Percentage digested starch of jasmine rice samples with and without additional 1% of hydrocolloid materials (carrageenan, pectin and gellan gum) during 90 minutes of stach hydrolysis process. Values are means of duplicates ± standard deviation.

3.4. In-vitro Bread Digestion

A preliminary work was conducted to adopt static *in-vitro* digestion on bread as a model sample. This work aims to predict digestibility and glycaemic index of different samples of bread compared to white bread sample.

To prepare the food samples, bread was cut into smaller sections (~1 cm of square). A 50 g of bread was processed using meat mincer for 5 minutes with addition of 50 ml SSF (no enzyme) to mimic oral mastication. Then bread bolus was mixed by hand to get more homogenious structure.

To start the gastric digestion phase, 10 g of the bolus was placed into a 50 mL corning or falcon tube. Simulated gastric fluid (SGF) containing 9.1 mL SGF stock, 5µl CaCl2, 696µl dH2O, 200µl 1M HCL (10 mL in total, pH3) was added the boluses. Then the mixtures was mixed for 30 min at 37°C using tube rotator in the 37°Cincubator.

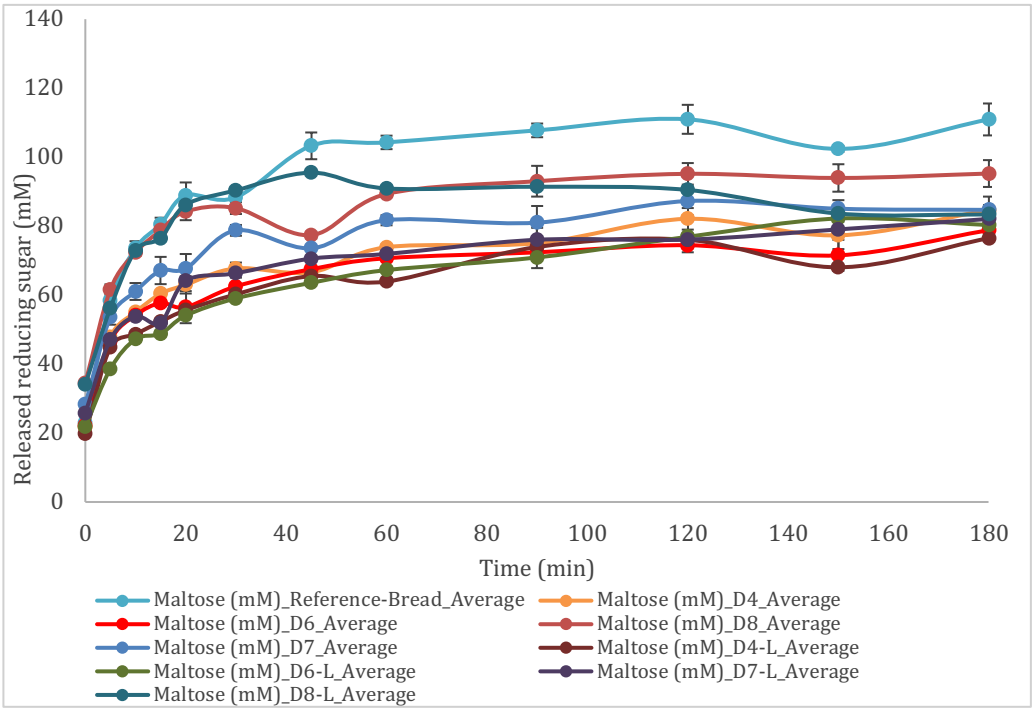
After the gastric digestion process, the simulated intestinal fluid (SIF) containing 14.5mL SIF stock, 150µl NaOH, 4µl CaCl2, 1.3mL dH2O (16 mL in total) was added to the chyme from the gastric phase. A 4 mL amylase working solution was added to the mixture to start the intestinal phase (start of the timer). The total volume of the intestinal phase was now 40 mL, with an amylase activity of (160U/mL x 4mL)/40 mL = 16U amylase/mL in the final digestion mixture. The tube was then placed back on a rotary mixer in the 37°C incubator.

The samples were then collected from the intestinal 'lumen' at 0, 5, 10, 15, 20, 30, 45, 60, 90 min (timed from the addition of pancreatic alpha amylase) by pipetting 200µl from the liquid phase into an equal volume (200µl) of 'STOP solution' 0.3M NaCO3 to instantly inactivates the amylase. Then the sample centrifuged at 12,500 rpm

(16 x G) using mini (test tube) centrifuge, and collect the supernatant into clean 1.5 mL centrifuge tubes. The supernatant was frozen at -20°C for later analysis. The next process was determination of reducing sugar using PAHBAH assay (Edwards *et al.*, 2018).

Batch 1 : Bread 'D' samples

In this experiment the different types of bread were tested which are part of the bread product development project. For the first batch, eight samples of bread which are divided into half of high density and another half of low density breads. As shown in the Figure 15, there are no significant different between low and high density bread on their digestibility and glycaemic index. All the D's bread product have lower GI value compared to commercial bread.



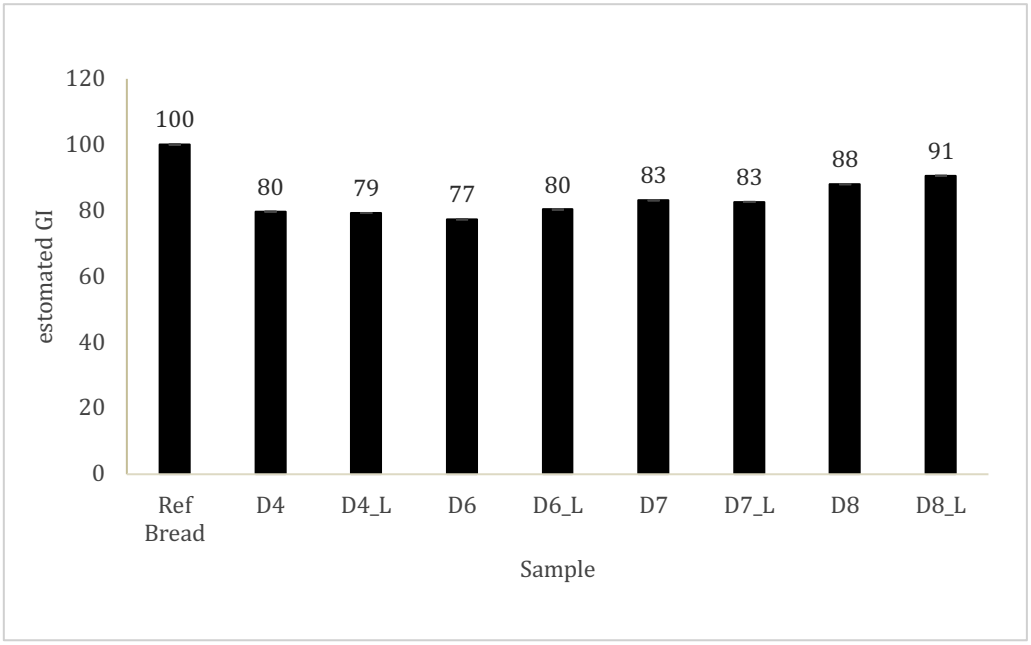


Figure 15. Released maltose equivalent (mM) of bread samples during static in-vitro digestion for 3 hour and its calculated glycaemic index value. The samples are D4; D6; D7; and D8 as high density bread products and D4_L; D6_L; D7_L; and D8_L as low density bread products, commercial white bread as reference. Values are means of triplicates ± standard deviation.

Batch 2: Bread 'E' samples

The next preliminary experiment on bread products was using Bread 'E" samples which are made with addition of dietary fibers and resistant starch. Figure 16 shows the released reducing sugar of the bread samples during invitro digestion for 3 hours and its estimated glycaemic index. All the Bread 'E' samples exhibit lower released reducing sugar and estimated glycaemic index. The Bread E4 sample shows the lowest digestible bread with 40% of decreasing in reducing sugar and 24% lower of glycaemic index value compared to bread reference.

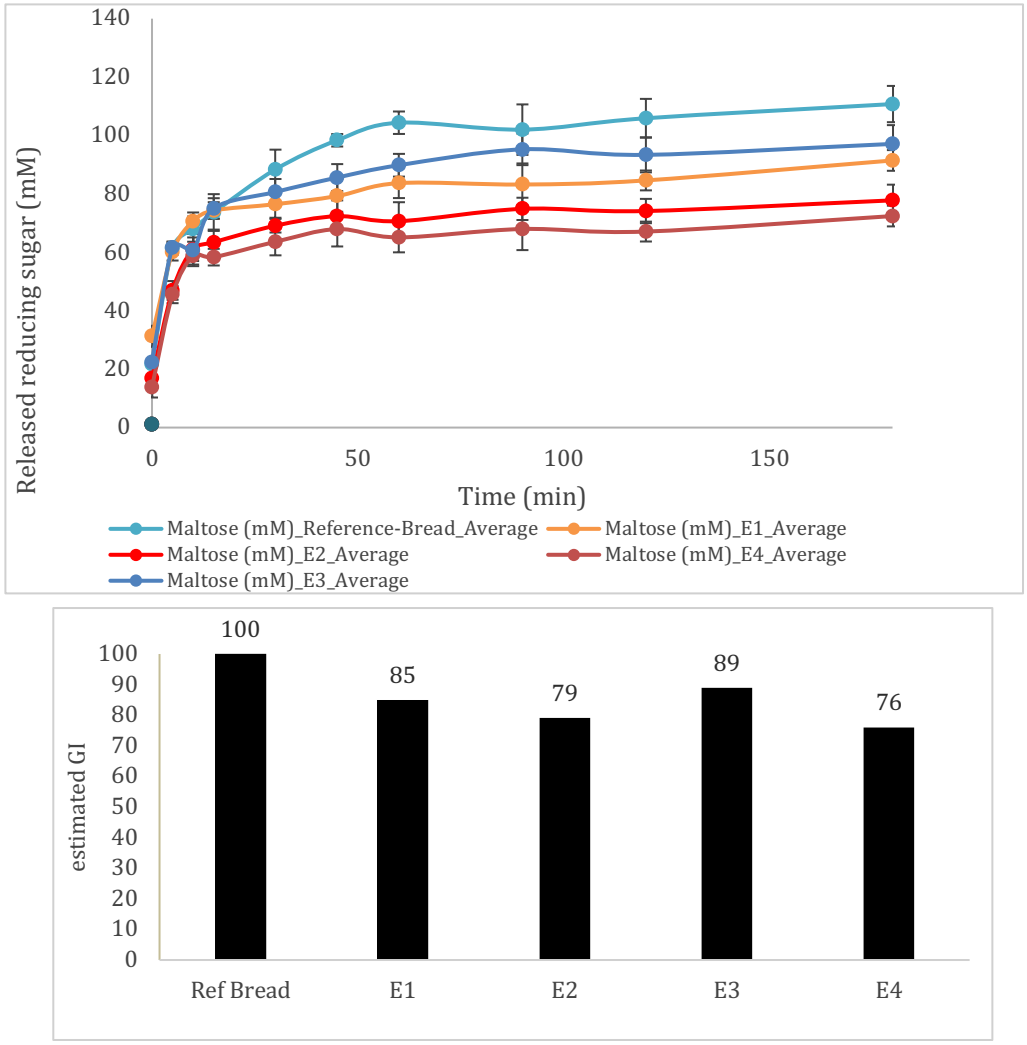


Figure 16. Released maltose equivalent (mM) of bread samples during static in-vitro digestion for 3 hour and its calculated glycaemic index value. The samples are 'E' Bread samples with different additional ingredients and commercial white bread as reference. Values are means of triplicates ± standard deviation.

Batch 3 : Bread 'A' sample

This batch 3 experiment was conducted to compare *in-vitro* bread digestibility between Bread 'A' sample and commercial white bread as reference. As one can see in the Figure 17, the released reducing sugar of bread 'A' sample was decreasing by 50% and its estimated GI value reduced for 28%. The results show that additional ingredient in bread 'A' could potentially slows digestion process, which can benefit as healtier bread product.

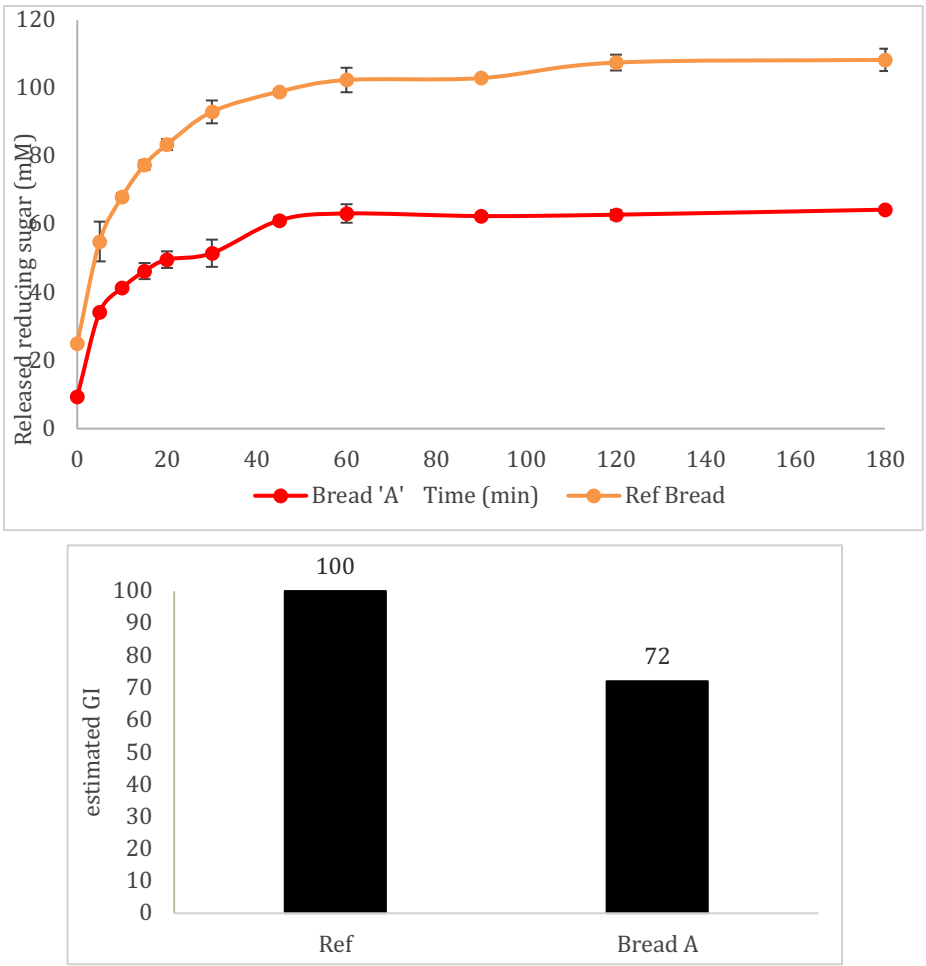


Figure 17. Released maltose equivalent (mM) of bread samples during static in-vitro digestion for 3 hour and its calculated glycaemic index value. The samples are 'A' Bread samples which made with additional ingredients and commercial white bread as reference. Values are means of triplicates ± standard deviation.

This preliminary studies shows that static in-vitro digestion can be used to measure food sample. The results indicate the differences bread digestibility with the different additional ingredient. Furthermore, glycaemic index of bread samples can be predicted with the calculation taken from Goni, Garcia-Alonso and Saura-Calixto, (1997). The static in-vitro digestion methods will be used for the next white rice digestion experiments.

CHAPTER 4

Reducing Starch Digestion of White Rice by Structuring with Hydrocolloids

Abstract

Rice is a staple food for >50% of the global population, consumed predominately in the form of polished, white rice. Whilst it provides an excellent source of carbohydrates, high consumption of white rice has been linked with adverse health effects such as increased risk for type-2 diabetes mellitus. In this work, addition of hydrocolloids during cooking of white rice has been considered as a means to reducing starch digestibility and therefore lowering the glycaemic response of the rice. Low acyl gellan gum (LAG) was identified as a potential candidate to reduce starch hydrolysis and lower the *in-vitro* estimated glycaemic index (eGI) of the rice by 20%. The rice prepared with LAG was harder but less sticky than the control without LAG. Visual observations (camera, cryo-SEM) suggested that LAG formed a (gelled) layer on the outer surface of the grain, which would be expected to strengthen at the low pH gastric environment. Mechanical breakdown during *in-vitro* oral processing resulted in similar size particles than in-vivo mastication. The presence of 1% LAG was further shown to obstruct disintegration of a simulated rice bolus in the stomach. During intestinal digestion, rice samples containing 1% LAG were less susceptible to α-amylase when seen under the microscope and in ESEM, while they showed larger rice particle aggregates, compared to rice control. Overall, LAG showed potential to control glycaemic response of white rice with a mechanism that involves formation of a protective gel layer at the rice grains' surface that reduces mechanical and enzymatic breakdown.

4.1. Introduction

Rice is a cereal staple food for more than half of the world's population (Muthayya *et al.*, 2014), and it accounts for about 20% of the total energy intake globally (Khatun, Waters and Liu, 2019). Around 84% of rice is consumed by Asian populations (IRRI, 2018), with China, India and Indonesia being the most rice consuming countries in the world.

The predominant type of rice consumed globally is polished (refined) white rice, which is produced from whole grain rice after extensive polishing (milling) processes that remove the husk, bran, and germ, leaving the starchy endosperm (Shobana *et al.*, 2011). White rice has higher organoleptic evaluations compared to brown or black rice (Zhong et al. 2014), however it has the majority of its dietary fibre, vitamins, and minerals, which are found in the outer layers of the rice kernel, has been removed (Shobana *et al.*, 2011). Altough rice is an excellent source of starch, however, high consumption of white rice has been linked with increased risk for adverse health effects such as obesity and type 2 diabetes (Hu et al., 2012; Nanri & Mizoue, 2014).

White rice contains about 80% starch (Saleh *et al.*, 2019), the exact amount depends on factors such as the rice variety and seasonal variability. Starch consists of two main polymers: amylose, a linear polysaccharide made of α-D-glycose units, and amylopectin, a highly branched polysaccharide of α-glucose units (Parada and Aguilera, 2011). During digestion, starch is hydrolysed mainly by the action of α-amylase, which is found in the saliva and in the pancreatic juices that are secreted during small intestinal digestion. A-amylase hydrolyses the linear regions of starch and converts the polysaccharide to low molecular weight products, predominately maltose and maltotriose, as well as dextrin containing the branching points of

amylopectin (Dhital *et al.*, 2017). Amyloglucosidase is further used to complete digestion and produce glucose molecules (Dona *et al.*, 2010). Starch digestion kinetics determine the rate of glucose release and absorption in the body (Bornhorst, Hivert and Singh, 2014). The terms rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) have been introduced by Englyst et al., (Englyst, Kingman and Cummings, 1992) to indicate the rate (and extent) of starch digestion. *In-vitro*, RDS and SDS are determined as the starch that has been hydrolysed after 20 and 120 min of intestinal digestion of the food; the remaining starch contributes the RS fraction (Englyst *et al.*, 2018).

The Glycaemic Index (GI) is a ranking system of starchy foods that indicates the rate of postprandial blood glucose changes after consumption of a food relevant to that of a reference material, usually bread or glucose. Typically, foods with high RDS have high GI. The GI of rice depends on factors such as the rice variety, starch type (amylose and amylopectin content), fibre content, gelatinisation history, cooling, soaking, germination, and particle size. (Kaur, Ranawana and Henry, 2016; Dhital, Dabit, Zhang, Flanagan, & Shrestha 2015; Srikaeo & Arranz-Martínez 2015). White rice generally has high GI (reported in the range of 24 and 160 (Kaur, Ranawana and Henry, 2016)), indicating high amount of RDS, while consumption of low GI rice has been suggested as a way to reduce the risk of type-2 diabetes incurred by the high consumption of high GI rice (Sar & Marks 2015). Efforts to reduce the RDS, hence the GI, have therefore been investigated; methods include annealing (Zeng, Chen, et al. 2015; Van Hung, Chau, & Phi 2016); fermentation (Zhang, Li, Chen, & Situ, 2016); stir frying (Kaur, Ranawana, Teh, & Henry 2015); soaking (Kale *et al.*, 2015); thermal treatment (Rattanamechaiskul, Soponronnarit, & Prachayawarakorn 2014)

retrogradation (Hsu, Chen, Lu, & Chiang 2015; Frei, Siddhuraju, & Becker 2003); and addition of oil (Kaur *et al.*, 2015)

Hydrocolloids have previously been associated with reduced starch digestibility and postprandial blood glucose levels, although their detailed role in digestion is not yet fully understood (Boers, Hoorn and Mela, 2016; Gidley, 2013). Suggested mechanisms include reducing or restricting contact between enzymes and substrates; reducing mass transfer rates in the gut (e.g., by increasing viscosity or gelling); and by inhibiting enzyme activity (Gouseti *et al.*, 2014; Boers, Hoorn and Mela, 2016; Gidley, 2013).

Addition of hydrocolloids to rice-based materials has been reported to affect the texture and digestibility of the food. Reduced *in-vitro* starch digestibility, compared to the control, has been found on addition of pullulan to rice starch (Chen et al. 2017); xanthan gum and guar gum to rice bread (Sasaki, 2018); and guar gum, xanthan gum, and sodium alginate to waxy rice (Srikaeo and Paphonyanyong, 2020). In vivo, coating rice with locust bean gum and agar decreased in starch digestibility in mice (Choi *et al.*, 2010).

In contrast, some studies that report an increase in starch digestibility when hydrocolloids are added to the material. Srikaeo et al., (2018) reported increased *in-vitro* starch digestibility of rice noodles in the presence of carboxymethyl cellulose (CMC), xanthan gum, and guar gum. One explanation given by the authors is that the hydrocolloids increased hydration of the noodle, resulting in increased digestibility. Similarly, Gularte & Rosell (Gularte and Rosell, 2011) reported higher starch digestibility when pectin, guar gum, and CMC were added to corn and potato starches, and they noted that the effect of the hydrocolloid was highly dependent on the exact nature of the hydrocolloid and the starch origin.

Gellan gum is a bacterial origin anionic polysaccharide that gels on cooling of hot solutions in the presence of gel promoting cations. Depending on the degree of acetylation, gellan gum is typically found in a high or low acyl form. It is a soluble dietary fibre that is added to foods, including rice, principally for texture modification (Fang *et al.*, 2018). Low acyl gellan gum gels are stronger, or can become stronger, at low pH (Bradbeer *et al.*, 2014; Norton, Cox and Spyropoulos, 2011; Cassanelli *et al.*, 2018). Gelation of LAG in the acidic environment of the stomach has been suggested as a potential mechanism to prolong satiety by stimulating the sense of fullness and prolonging gastric emptying (Norton, Cox and Spyropoulos, 2011; Bradbeer *et al.*, 2014).

The present work aims to study the effect of hydrocolloid addition during cooking on the structure and *in-vitro* digestibility of white rice. From the investigated hydrocolloids LAG showed the greatest potential to reduce starch digestibility in white rice and was further studied.

4.2. Materials and Methods

4.2.1. Materials

Jasmine rice (Double Elephant, Thailand) and white bread (Kingsmill, UK) were purchased from a local supermarket. Gellan gum was obtained from CPKelco, UK. All other materials were supplied from Sigma-Aldrich, UK and were of analytical grade except otherwise stated.

4.2.2. Methods

4.2.2.1. Rice sample preparation

Rice was cooked based on the manufacturer's instructions. For the preparation of rice without any hydrocolloids, 100g of rice were cooked in a commercial rice cooker (Cookwork, UK) with 150 mL of distilled water. Rice was cooked in the automatic 'cook' mode for 14 min and it was then left in the 'warm' mode for 6 min.

For the preparation of rice with added hydrocolloids, the required amount of hydrocolloid to produce the concentrations shown in Table 2 was first dissolved in 150 mL of distilled water at 80°C for 20 min in a beaker covered with aluminium foil under gentle mixing (magnetic stirrer) using a hot plate. Then, 100 g of rice were added and the mix was transferred and cooked in the rice cooker as described in the previous paragraph (i.e., 14 min in 'cook' and 6 min in 'warm' mode).

Table 2. Investigated rice preparations without and with hydrocolloids

Hydrocolloid added	Hydrocolloid concentration (%w/w of uncooked rice)
n/a	n/a
Low Acyl Gellan Gum	0.5%
Low Acyl Gellan Gum	1%
High Acyl Gellan Gum	1%
Pectin	1%
Gum Arabic	1%

Cooked rice with or without hydrocolloids, was transferred to a 500 mL beaker after the 6 min in 'warm' mode and it was immediately covered tightly with aluminium foil to prevent water evaporation.

For the *in-vitro* digestion experiments, the samples were prepared at the same day of the experiment and they were kept in the beaker at room temperature for an additional 10 min before starting oral digestion. By that time, the temperature of the rice preparations was about 35°C. The investigated preparations are summarised in Table 3.

4.2.2.2. In-vitro Digestion of Cooked Rice Preparations

Preparation of digestive solutions

The *in-vitro* digestion protocol was based on previous studies (Minekus *et al.*, 2014; Brodkorb *et al.*, 2019) with some modifications. Three stages of *in-vitro* digestion were performed: oral, gastric, and small intestinal. In each of the three stages, simulated digestive fluids were added: simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) for the oral, gastric, and small intestinal phases, respectively. For the gastric and small intestinal digestion, the

relevant Enzymatic Fluids (Gastric Enzymatic Fluid, GEF, and Intestinal Enzymatic Fluid, IEF) were added to the digestion experiment as part of the final SGF and SIF.The electrolyte as well as enzyme concentrations in these fluids are shown in Table 3.

Table 3. Electrolyte and enzyme concentrations in the simulated digestive fluids

Digestive fluid	Electrolyte concentrations	Enzymes & concentration
SSF	15.1mM KCl, 3.7mM KH_2PO_4, 13.6mM $NaHCO_3$, 0.15mM $MgCl_2(H_2O)_6$, 0.06mM $(NH_4)_2CO_3$, 1.1mM HCl, 1.5mM $CaCl_2$	n/a
SGF	6.9mM KCl, 0.9mM KH_2PO_4, 25mM $NaHCO_3$, 47.2mM NaCl, 0.12mM $MgCl_2(H_2O)_6$, 0.5mM $(NH_4)_2CO_3$, 15.6mM HCl, 1.15mM $CaCl_2$	2000U/mL pepsin from porcine mucosa
SIF	6.8mM KCl, 0.8mM KH_2PO_4, 85mM $NaHCO_3$, 38.4mM NaCl, 0.33mM $MgCl_2(H_2O)_6$, 8.4mM HCl, 0.6mM $CaCl_2$	24U/mL porcine pancreatic amylase

Stock electrolyte solutions (Stock SGF and Stock SIF) were prepared in advance and were added to the digesta separately to the enzymes, as the other part of the SGF and the SIF. The enzymes for the SGF and SIF were added to the digestion experiment shortly after dispersing them in distilled water. Each simulated digestive fluid, therefore, consisted of the relevant stock solution and enzymes (i.e., SGF consisted of Stock SGF and GEF; SIF consisted of Stock SIF and IEF). The electrolyte and the enzyme concentrations in the fluids were such as to ensure final concentrations in the SGF and SIF as shown in Table 2. Each digestion experiment required 8.4mL of Stock SGF and 1.6 mL of GEF in the gastric phase. To ensure that all the pepsin was added in the experiment, the required amount of powder was dispersed in 1mL of distilled water; this was added in the digesta and the container was rinsed with additional 0.6mL of distilled water that were then added to the experiment. For the intestinal phase, 16 mL of Stock SIF and 4mL of IEF were added. The enzyme was dispersed in

3mL of distilled water and the container was rinsed with the remaining 1 mL of distilled water, which was also added in the digestion experiment.

The targeted pH for the three investigated stages of digestion was 7 for the oral, 2 at the beginning of the gastric, and 7 at the beginning of the small intestinal digestion. An initial 'blank' (i.e., without measuring starch hydrolysis) digestion experiment was carried out to determine the amount of 1.0M HCl required in each stage to adjust the pH to the targeted value. This was then added in the preparation of the Stock solution of the relevant simulated digestive fluid. This step was deemed necessary to include the volume of the required HCl in the calculations of electrolyte concentrations. The pH of the mixture of chyme entering the small intestinal digestion and SIF was 7 and so no adjustment was required at this stage.

In-vitro digestion

In-vitro starch digestion was carried out according to Brodkorb *et al.*, (2019), Minekus *et al.*, (2014) and Edwards *et al.*, (2018) with some modifications. In brief, for each digestion, 5g of the rice preparation were mixed with 5mL of SSF in a mini chopper (Kenwood, UK) for 20s (with 5s circles of 4s mixing and 1s pausing). The resulting bolus was transferred into a falcon 50mL tube and was vortex mixed (2s) with 10mL SGF (8.4mL of Stock SGF + 1.6mL of GEF, as described above). The tube was placed in a rotator and the rotator was placed in a 37°C incubator. Gastric digestion was conducted for 30min at 10rpm. 16mL of Stock SIF were then added to the resulting chyme, and the sample was placed back on the rotating rotator for mixing. An aliquot of 200μl was taken as the 0-minute sample. To begin starch hydrolysis, 4mL of IEF were added to the tube and the tube was placed back on the rotating rotator. Intestinal digestion was conducted in the rotator at 10rpm, 37°C for

180min. During the intestinal digestion, aliquots of 200μL were taken at each time point (5, 10, 15, 20, 25, 30, 45, 60, 90, 120 and 180 minutes) and were immediately placed into a 1.5mL test tube containing 200μL of stop solution (0.3M Na_2CO_3). This process has been previously used to stop the enzymatic hydrolysis by increasing the pH of the system (to approx. 11) (Edwards *et al.*, 2018). The aliquots were centrifuged (12500 rpm, 5 minutes, mini centrifuge Fisher Scientific, USA) and 20μL from the supernatant were collected and diluted appropriately to reach the sugar concentration range of the analysis.

The concentration of reducing sugars in the diluted samples was determined using the 4-Hydroxybenzhydrazide (PAHBAH) assay. First, the PAHBAH solution was prepared freshly before the analysis by dissolving 250mg of PAHBAH powder in 4.75mL of 0.5M HCl using a vortex in a 50mL falcon tube, then vortex mixing it with 45mL of 0.5M NaOH. 1mL of the freshly prepared PAHBAH solution was vortex mixed with 100μL of a (diluted) sample in a micro test tube. The tube was then placed in a water bath at 95^0C for 5 minutes and it was then left on the bench to cool to room temperature (circa 25°C). Absorbance at λ=405nm was compared to a calibration curve. The calibration curve was produced using maltose as a reducing sugar, as this is known to be the main product of the starch hydrolysis with α-amylase (Dhital *et al.*, 2017).

4.2.2.3. Characterising structure of rice grains before digestion

Images of whole rice grains with and without LAG, before subjecting them to *in-vitro* digestion, were acquired using a DSLR camera (Canon 5D, 64 mm macro lens) and the regular lighting sources of the laboratory (room lights). Microstructure of the

rice preparations before *in-vitro* digestion was examined using Cryo-SEM (Philips XL30 ESEM, Japan). Samples were first frozen in liquid nitrogen and were kept in the preparation chamber under vacuum at -90^{0}C for 15 minutes before being cut and coated with gold. Coated samples were transferred in the SEM chamber for visualisation (5kV power).

Cooked rice was further examined for its textural properties using a TA.XT-Plus Texture Analyzer (Stable Micro Systems Ltd, Surrey, UK) and methods based on Texture Profile Analysis (TPA) (see for example Li *et al.*, 2016). 4 grains of cooked rice without and with 1% LAG were placed at the centre of the texture analyser base plate. The samples underwent a two-cycle compression test with 80% strain using the 36mm cylindrical probe attachment at a 1mm/s speed. Hardness and stickiness (or adhesiveness) were determined from the force vs time curve as the highest peak force during the first compression and the area of the first negative peak, respectively. 6 replications of each experiment were conducted and mean values with standard deviations are reported.

4.2.2.4. Effect of oral processing on *in-vitro* digestion

Particle sizes of rice samples without and with 1% LAG that have been subjected to the oral processing described in Section 4.2.2.2. were compared to those of in-vivo masticated rice samples. 15 cycles of mastication were performed for each sample, which produced a bolus ready to swallow. Particle size was measured by analysing images of the samples acquired using an EVOZ light microscope.

The effect of mechanical breakdown during oral processing on rice digestibility was then examined for rice without hydrocolloid and rice with 0.5% and 1% low acyl

gellan gum. For each sample, two sets of *in-vitro* digestion experiments were conducted, one following the procedure described in section 4.2.2.2, and the other omitting the use of the mini chopper during simulated oral digestion. In the latter experiments, the rice samples were vortex mixed with the required amounts of SSF and SGF in a 50mL falcon tube before starting the 30min of gastric digestion in the rotator at 37°C, 10rpm. Data from these experiments were used to calculate the estimated Glycaemic Index (eGI) of each digested sample. For the calculations, the starch hydrolysis curves were fitted to equation (4)

$$C = C^{\infty}(1 - e^{-kt}) \quad (4)$$

where C is the percentage of starch that has been hydrolysed at each time point; C^{∞} is the maximum percentage of starch hydrolysed after 2h of small intestinal digestion; k is the kinetic constant; and t is the time. Equation (4) was further used to calculate the area under the curve for each sample (using the trapezoid rule, see Englyst *et al.*, 2003). Digestion experiments were also carried out for white bread (Kingsmill white bread), which served as the reference material. The Hydrolysis Index (HI) of each of the digested samples was calculated as the area under the hydrolysis curve of the sample divided by the area under the curve of the reference, which was taken from the area under curve of white bread, thus eGI was calculated using equation (5) (Wolter *et al.*, 2013).

$$eGI = 39.71 + 0.549HI \quad (5)$$

4.2.2.5. Rice Bolus Disintegration at simulated gastric digestion conditions

Rice boluses were prepared to study the effect of gellan gum on bolus disintegration under simulated gastric digestion conditions. The amounts of liquid used in these experiments were such as to ensure formation of a coherent bolus. 50g of rice cooked without and with 1% LAG, prepared as described in section 4.2.2.1, were blended for 20 seconds with 25mL Phosphate Buffer Saline (PBS, Sigma, UK) using a mini food processor (Kenwood, UK). 1mL of Human Salivary Amylase (75U/mL) was added to the blend and it was further mixed by hand for 2 minutes to create the bolus material. 6g of the bolus material were taken and made to a spherical shape (2.5cm diameter) using a spherical mould.

To monitor disintegration of the material, the spherical bolus was hung from its central point at one end of a wire. Using the other end of the wire, it was then placed carefully at the centre of a 500mL glass chamber to avoid contact with any of the chamber's walls. The glass chamber had a black background on the side opposite to the visualisation window and a cubical shape so as to enhance image quality (see Figure 18). A high-resolution camera (Canon DSLR 5D, 24-100 mm lens with 31 mm macro lens extender) and dedicated lighting (Aputure, Amaran AL-528W lights with 90% light intensity) were used for the recordings. The setting and positions of the sample, lighting, and camera were kept identical for all experiments. After the bolus was hung in the centre of the chamber, video capturing was started, and 500 mL of PBS pH 2 was added gently into the chamber using a tube connected to a syringe. Recordings continued for 1 hour as the bolus disintegrated into the liquid. The video was then converted into frames and the normalised radius of the remaining bolus at different times was determined using Matlab.

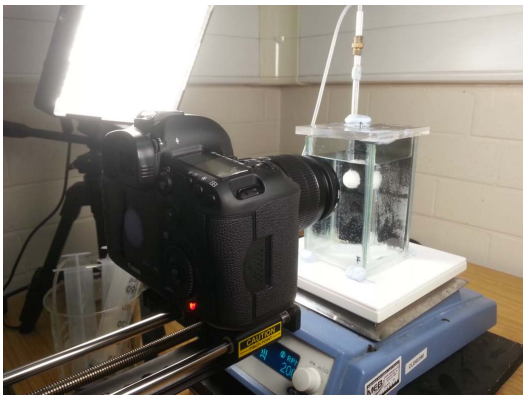


Figure 18. Experimental setup for monitoring bolus disintegration under simulated gastric environment.

4.2.2.6. Structural changes during in-vitro intestinal digestion

Two separate experiments were conducted to study the structural changes during *in-vitro* small intestinal digestion.

In the first experiment, thin slices (approximately 1 mm thick) were carefully cut by hand with a blade knife (as in Tamura *et al.*, 2014) from the centre of single cooked rice grains without and with 1% LAG to produce circular cross-sections, which were then cut into two hemi-circles. A single hemicircular sample was placed on one well of a 96-well plate and the plate was mounted onto the visualisation window of an EVOZ light microscope to cover its full area. SIF containing 24U/mL pancreatic amylase was added to the rice sample, then a single drop of Lugol Iodine solution was added. Image recording (at 10x magnification) started immediately and continued for 30 minutes. Images were captured every 10 seconds for 30 minutes to produce a 3-minutes time lapse video (shown in the additional material). It was noted that images acquired with samples that had passed through oral and gastric digestion were not sharp enough for visualisation.

In the second experiment, structural changes occurring during digestion were observed in the liquid and solid parts of the material. For the liquid parts, rice samples without and with 1% LAG were subjected to the *in-vitro* digestion procedure described in section 4.2.2.2 and aliquots of 200μL were visualised before and after the *in-vitro* small intestinal digestion under an EVOZ light microscope. For the solid parts, chunks of rice present in the samples before any digestion step and after small intestinal digestion were collected and analysed using ESEM (FEI Quanta 650, Thermo Scientific, UK) after being mounted on the ESEM stub with conducting carbon cement. Non-digested rice samples were stored in the refrigerator over-night, until the next ESEM scanning session, and were analysed at 95% humidity. Digested samples were first soaked for 1min in the stop solution (0.3M Na_2CO_3) which stops the enzymatic reaction, were then transferred to Whitman filter papers to remove excess water, and then kept in small plastic containers in the fridge overnight, until the next day's ESEM analysis. These samples were analysed at 75% humidity.

4.3. Results and Discussions

The following results are presented and discussed in this section: first, the in-vitro digestibility profiles of rice without or with different hydrocolloids added (at 1% concentration); then, the cooked rice grain's structure without and with 1% LAG; the effect of oral processing on their digestibility profiles; bolus disintegration in simulated gastric digestion environment; and their structural changes during simulated small intestinal digestion.

4.3.1. *In-vitro* digestibility of cooked rice with additional hydrocolloids

Rice preparations containing 1% of low acyl gellan gum, high acyl gellan gum, gum arabic, and pectin, as well as control rice without added hydrocolloids were subjected to in-vitro digestion. Selection of hydrocolloids was based on their properties and the fact that all are commonly used in foods and have been previously used in in-vitro digestion studies. Gum arabic is a highly soluble thickener, whereas pectin and gellan gum are gelling agents. (Saha and Bhattacharya, 2010).

Figure 19 shows the obtained starch hydrolysis curves of the samples during the in-vitro small intestinal digestion. For all conditions, starch hydrolysis rate was initially high and reduced with time as expected (see e.g., Goñi, Garcia-Alonso and Saura-Calixto, 1997). Under the investigated in-vitro digestion conditions, the presence of 1% gum arabic, pectin, and high acyl gellan gum appeared to have marginal effect on starch digestibility. Starch hydrolysis curves were comparable to that of the rice control sample that resulted in about 90% of the starch being hydrolysed

after 180min of small intestinal digestion. Interestingly, 1% low acyl gellan gum seemed to reduce starch hydrolysis rate and the final value of hydrolysed starch was 30% lower, reaching 60% of the total initial starch.

The effect of hydrocolloids on digestion is an active area of research. Previous studies have reported that the presence of gum arabic may reduce starch digestibility in noodle preparations (Bae et al., 2019) or marginally affect protein digestibility of milk proteins (Mouécoucou et al., 2003). Pectin is a hydrocolloid that gels on cooling under acidic conditions. Depending on the nature and source of the pectin, it has been reported that it may have a lowering or little effect on starch digestibility, for example by inhibiting the action of α-amylase or increasing viscosity of the digesta (Sasaki, Sotome and Okadome, 2015; Bai et al., 2021). Interestingly, pectin has also been reported to enhance starch digestion, attributed to the interactions between the particular pectin used, starch source, and conditions (e.g., Gularte and Rosell, 2011).

Gellan gum gels on cooling in the presence of gel-promoting cations, and gel strength has been reported to depend on the pH during gelation, with maximum gel strength obtained at pH 3 (Bradbeer et al., 2014; Norton, Cox and Spyropoulos, 2011; Cassanelli et al., 2018). Exposure of already formed gellan gum gels to low pH has also been reported to increase gel strength, with low acyl gellan gum gels being more sensitive to postproduction acid exposure compared to high acyl gellan gum gels (Bradbeer et al., 2014).

The observed reduction in starch hydrolysis for the rice cooked in the presence of 1% LAG, may therefore be associated with the formation of a gel layer on the rice grain during cooling, which is then strengthened as the material passes through the acidic environment of gastric digestion. As the chyme enters small intestinal digestion, the presence of a strong gel may reduce starch digestion.

The aim of these initial experiments was to select the best performer from the investigated hydrocolloids, and LAG was identified as a potential candidate to reduce starch hydrolysis rate of rice. As such, in the following sections, the effect of low acyl gellan gum on rice digestion will be further examined.

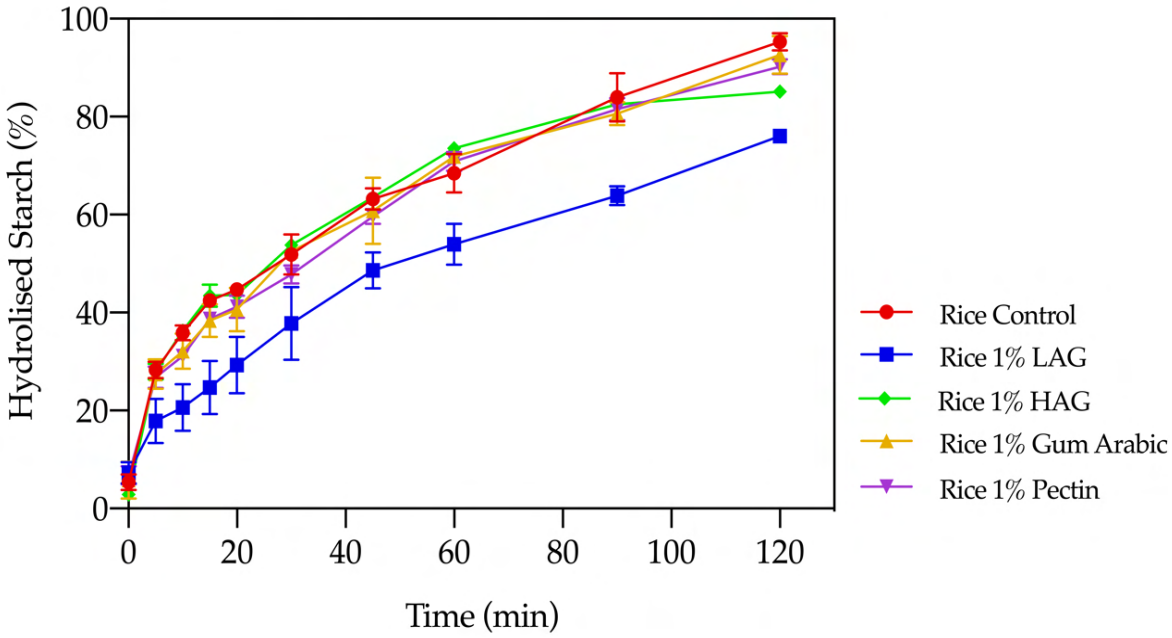


Figure 19. Starch hydrolysis curves during in-vitro small intestinal digestion for cooked rice without hydrocolloids and rice with 1% low acyl gellan gum (LAG), 1% high acyl gellan gum (HAG), 1% gum arabic, and 1% pectin. Values are means of triplicate ± the standard error of the mean (SEM).

4.3.2. Structure of Rice with addition of gellan gum

Figure 20 shows the structure of rice cooked without and with 1% LAG observed with a DSLR camera and cryo-SEM. For comparison, the preparation with 1% high acyl gellan gum is also shown. High acyl gellan gum is known to form softer and more elastic gel structures compared to those of low acyl gellan gum (Sworn, 2009).

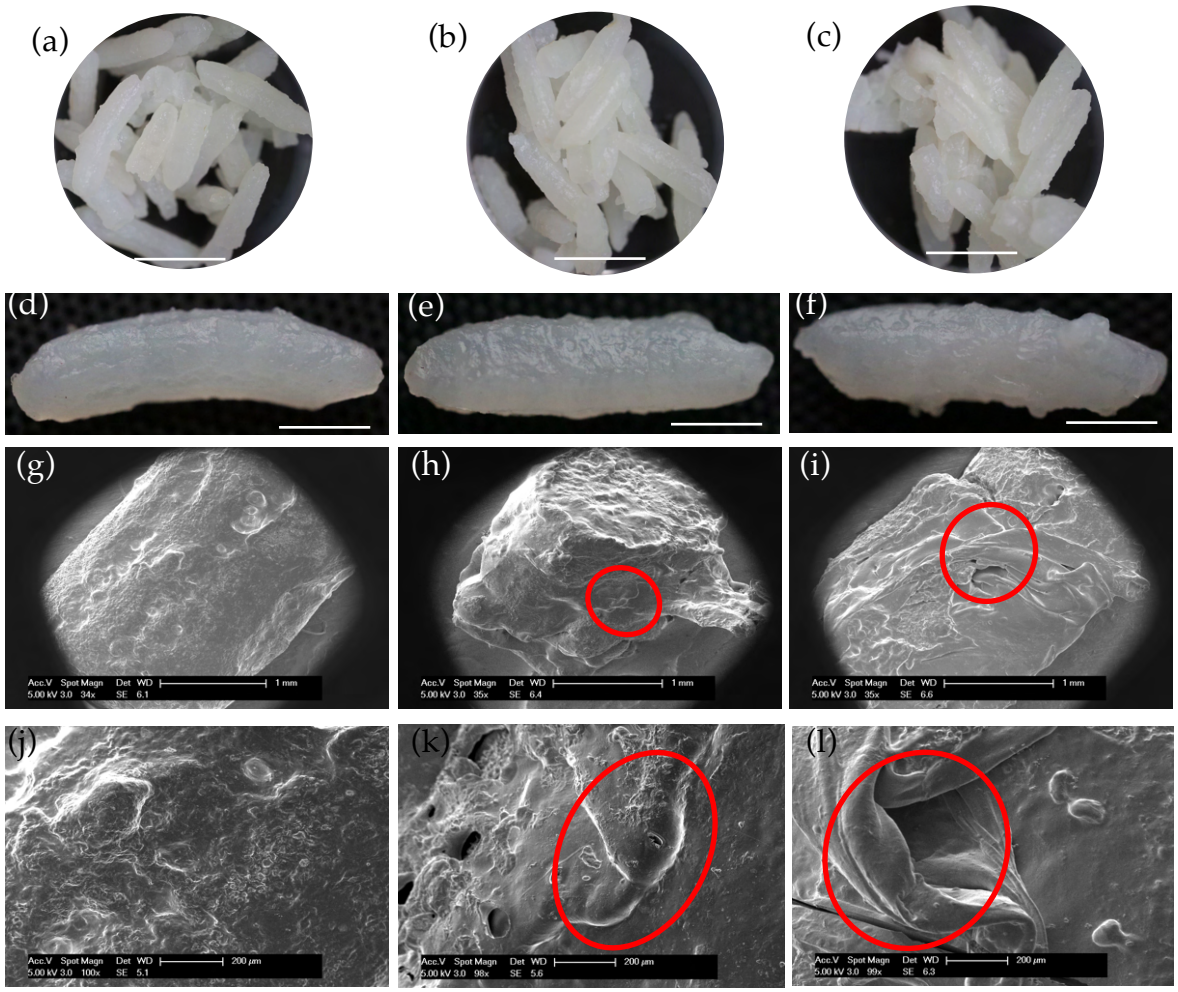


Figure 20. Representative structure images of cooked rice (a, d, g, j), rice with 1% low acyl gellan gum (b, e, h, k), rice with 1% high acyl gellan gum (c, f, i, l). Bars on pictures a-e represent 2.50 mm. Microstructure of cooked rice surface by Cryo SEM shows presence of a gelled layer covering the surface of the rice grain (g-l, regions enclosed in red lines)

Figures 20a-20c indicate there is little effect of gellan gum on the appearance of cooked rice grains, although the rice appears to form clusters in the presence of the hydrocolloid, possibly due to the formation of gellan gum gel that acts as a glue between different rice grains. The surface structure of the rice grains without and with hydrocolloid, Figures 20d-20f, appear similar, although the presence of gellan gum resulted in thicker grains (i.e., with larger cross sections), which could be linked with the formation of a thin gel layer surrounding the surface of the grain.

Formation of a gel layer on the surface of the rice grains when cooked in the presence of gellan gum is also confirmed in the cryo-SEM images of Figures 20g-20l (as indicated in the areas enclosed by red lines/circles). Rice was cooked without any excess water, therefore all the added amounts of water and gellan gum were absorbed by/deposited on the rice grains, allowing for gel formation during cooling.

The texture profile of the cooked rice was also investigated. Hardness and stickiness are two key parameters of cooked rice texture quality and have opposite correlation (Li *et al.*, 2016). Figure 21 shows the obtained hardness and stickiness values for the rice preparations without and with 1% LAG. Hardness increases in the presence of the hydrocolloid, but the difference was not significant ($P>0.05$). This trend may be due to the formation of a firm and brittle gel as the low acyl gellan gum sets during cooling (Sworn, 2009). On the other hand, stickiness significantly decreases ($P<0.05$) when the rice was cooked with LAG. Stickiness in cooked (jasmine) rice has been attributed to amylose and (short chain) amylopectin molecules that leach out of the grain during cooking and glue the grains together (Li *et al.*, 2016; Leelayuthsoontorn and Thipayarat, 2006). It appears that the presence of LAG reduces molecular leaching from the grain and/or the ability of any leached molecules to attach to the intact grains.

This is probably related to the covering of the rice grains with gellan gum that sets during cooling.

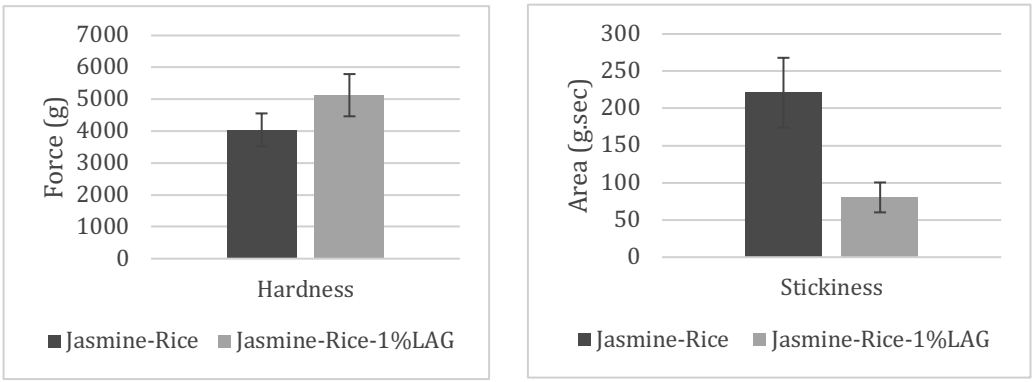


Figure 21. Texture properties of Cooked Jasmine rice control and with addition 1% gellan gum measured using Texture Analyzer. The hardness and stickeiness were determined from the force vs time curve as the highest peak force during the first compression and the area of the first negative peak, respectively. Values are means with standards deviation of 6 replications Statistical significance determined using the Holm-Sidak method, with alpha = 0.05. Each row was analyzed individually, without assuming a consistent SD. Number of t tests: 2.

4.3.3. Effect of oral processing

Figure 22 shows representative images of rice samples without and with 1% LAG that have been masticated (labelled as "masticated") or undergone oral processing as described in section 4.2.2.2 (labelled as "chopped"). Particle area of the chopped and masticated rice samples was determined at 8.6±3.9mm^2 and 6.4±2.2mm^2, respectively. For the rice with 1% LAG, the surface area of chopped and masticated samples was 9.7±3.3mm^{2}and 7.3±2.7mm^2, respectively. It appears that mastication created smaller particles compared to chopping, and the presence of 1% LAG resulted in larger particles compared to rice control, however the differences were insignificant.

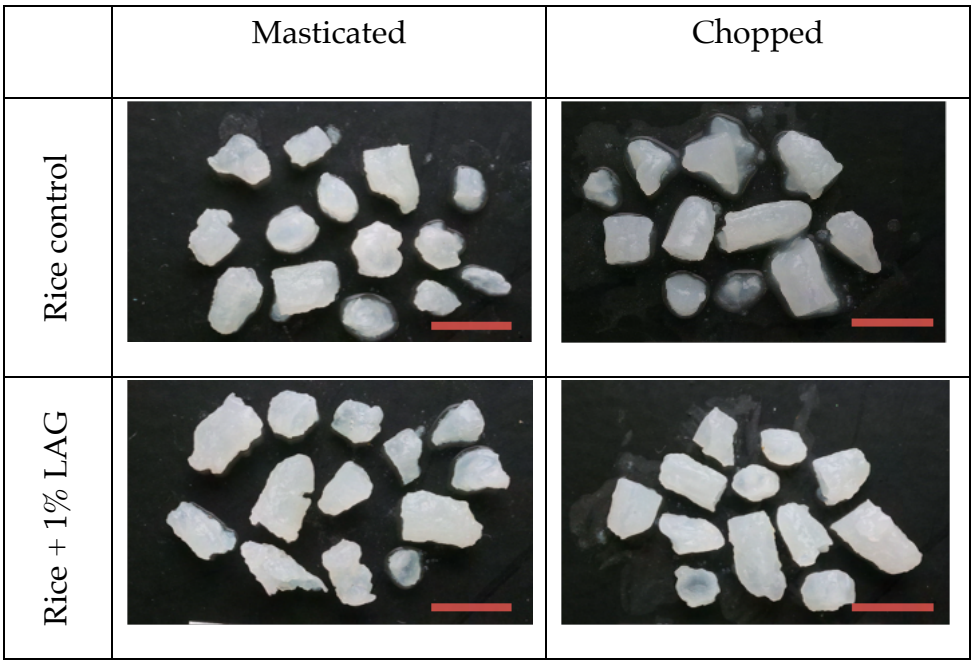


Figure 22. Representative images of rice samples without (top row) and with (bottom row) 1% LAG that have been masticated (1st column) or undergone in-vitro oral processing using a mini chopper (2nd column). Red scale bar represents 5 mm.

The effect of mechanical breakdown during oral digestion on the in-vitro starch digestibility of rice was further investigated at LAG concentrations of 0, 0.5, and 1%. Samples underwent in-vitro digestion with and without mechanical breakdown using the mini chopper in the oral phase. The resulting starch hydrolysis curves are shown in Figure 23a. Data from figure 6a have been used to calculate the estimated Glycaemic Indices (eGI) of the investigated rice samples (Figure 23b). It is noted that the eGI is an in-vitro determined value characteristic of a starchy food that indicates how slowly or quickly starch is being digested from this food relative to a reference material (usually glucose or white bread). It is used as an in-vitro ranking tool between different starchy foods, and it can potentially facilitate prediction of the in-vivo glycaemic index of the foods. GI of starchy foods such as rice may further depend on the cooking history of the food, for example it has been suggested that addition of oil before or after cooking may affect the rate and extend of starch digestion (Kaur *et al.*, 2015).

For all investigated systems, Figure 21 indicates that mechanical size reduction of the sample during the oral step resulted in increased starch digestibility. This was expected, as smaller particles are expected to digest faster than larger particles due to the higher surface area/volume ratio, resulting in higher exposure of the material to the digestive fluids. Tamura *et al*. (2014) studied the effect of structure and particle size on digestibility of rice and reported that homogenised rice samples digested 8 times faster than intact rice grains.

For the rice samples that were digested intact, reducing LAG concentration from 1 to 0.5% had marginal effect on the starch hydrolysis curve of the food, and the two curves overlapped. By contrast, when samples were mechanically broken during the oral phase, the starch hydrolysis curve of the 0.5% LAG rice sample was comparable to that of the control rice sample.

It has previously been suggested that LAG does not form a gel at 0.5% concentration at its natural pH (around 5.4), however a gel is formed at acidic environment, e.g., pH<3.5 (Cassanelli, Norton and Mills, 2018; Bradbeer *et al.*, 2014). At 0.5% concentration, LAG may form a protective (non-gelled) layer on the rice grain that, if left intact, reduces interactions of the starch with the digestive enzymes, resulting in lower starch digestibility. When subjected to the acidic gastric environment, the protective layer may further form a gel that enhances its protective activity. Mechanical breakdown appears to disrupt this non-gelled protective layer and expose the food material to the digestive enzymes. At 1% concentration, a gel is being formed during rice preparation that remains in place during oral mechanical breakdown. The sides of the rice grain that have been covered by the gel layer remain protected, and the overall result is a reduction in starch digestibility both in intact and chopped rice.

The eGI values of Figure 23b were calculated using white bread as reference and complement the observations from the curves in Figure 23a. Samples that showed reduced starch digestibility exhibited lower eGI values. The eGI of white jasmine rice control was 94, which is within the range suggested for Thailand jasmine Rice porridge (68-97), albeit the authors used glucose as reference (Srikaeo and Sopade, 2010). Addition of 1% LAG to the rice resulted in approximately 20% reduction on the eGI (from 94 to 78 for the rice that underwent full in-vitro digestion), indicating the potential for milder postprandial glucose response.

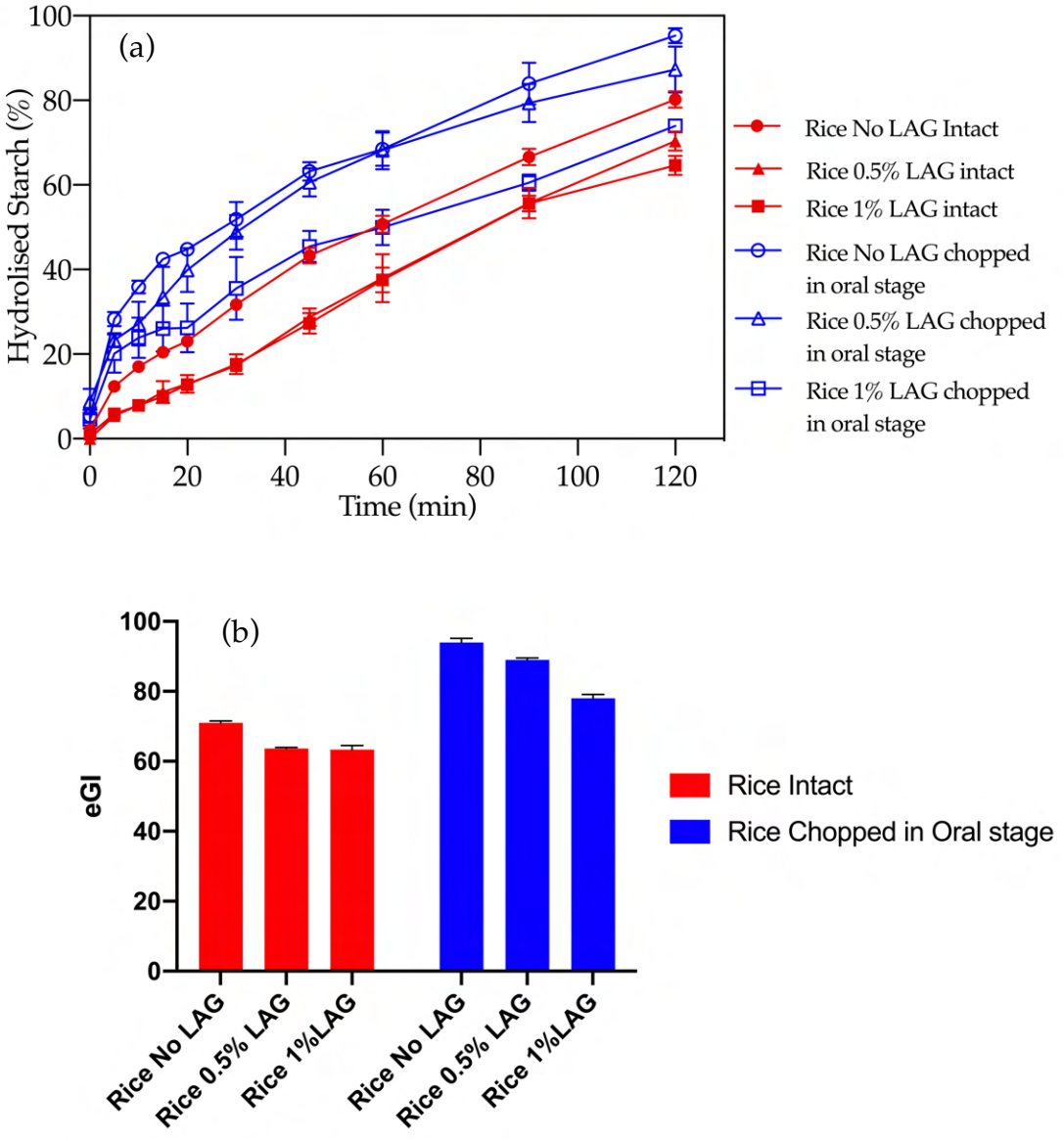


Figure 23. Starch digestion of rice at LAG concentrations of 0, 0.5, and 1%, without (intact) and with mechanical breakdown during oral digestion: (a) starch hydrolysis curves of small intestinal digestion; and (b) estimated Glycaemic Indices of the investigated samples using white bread as reference. Values are means of triplicate ± the standard error of the mean (SEM).

4.3.4. Rice bolus disintegration

Bolus disintegration indicates the rate and extent of food breakdown into smaller fragments in the stomach. It is key in controlling gastric emptying (particles broken down to abound 2mm can be emptied through the pylorus to enter the duodenum) and in determining particle size distribution in the chyme that passes to the small intestine for further digestion (Kong and Singh, 2008; Bornhorst, Kostlan and Singh, 2013).

Disintegration of spherical rice boluses without and with 1% LAG was observed under simulated gastric conditions and changes in the radius of the boluses with time, together with selected images, are shown in Figure 24. In Figure 24a shows that the rice bolus without hydrocolloid first swelled, suggesting that liquid is being absorbed, then disintegrated through a mechanism that involved the removal of large pieces of bolus material from the initial sphere (indicatively, the first large piece removal caused an abrupt 33% radius reduction – corresponding to 30% volume reduction). The size of the falling pieces gradually reduced, causing smaller radius reductions. After 10min, the bolus was about half its initial size, and after 30min it had fully disintegrated and the minimum radius was achieved, that of the hanging wire's hook. The observed disintegration mechanism may be attributed to the hydrolysis of starch within the bolus by the action of salivary amylase, present in the material from the oral phase. This may be partially facilitated by the diffusion of water, which may relax the cohesive forces in the bolus - a 20% increase in radius was observed after 2min of immersion of the bolus to the fluid. However, it should be noted that diffusion of water further prompts penetration of acid in the material, and the resulting pH reduction will gradually inactivate amylase. It has been previously suggested that the

salivary amylase present in a bolus can be active in the stomach until the pH gradually drops to inactivation levels by the penetration of gastric acid (Mennah-govela, Bornhorst and Singh, 2015).

Figure 24b illustrates the protective effect of 1% LAG in the disintegration of the rice bolus in the presence of the hydrocolloid. In this case, the bolus appeared to gradually absorbed liquid and swelled, reaching a 10% increase in the radius after 1h of observation. Under investigated conditions, no obvious disintegration was recorded within the experimental time. At 1% concentration, LAG can form a gel on the rice grain, protecting it from digestion. Under low pH, such as the acidic environment of gastric digestion, LAG gels are expected to strengthen (Bradbeer et al., 2014; Norton, Cox and Spyropoulos, 2011), further enhancing the protective effect. It appears that the gel both caused slower water diffusion in the bolus (as without the LAG, bolus radius increased by 20% in 2min) and prevented the removal of material from the bolus. It is anticipated that salivary amylase was still active within the bolus, however the acidified LAG gel held the material together.

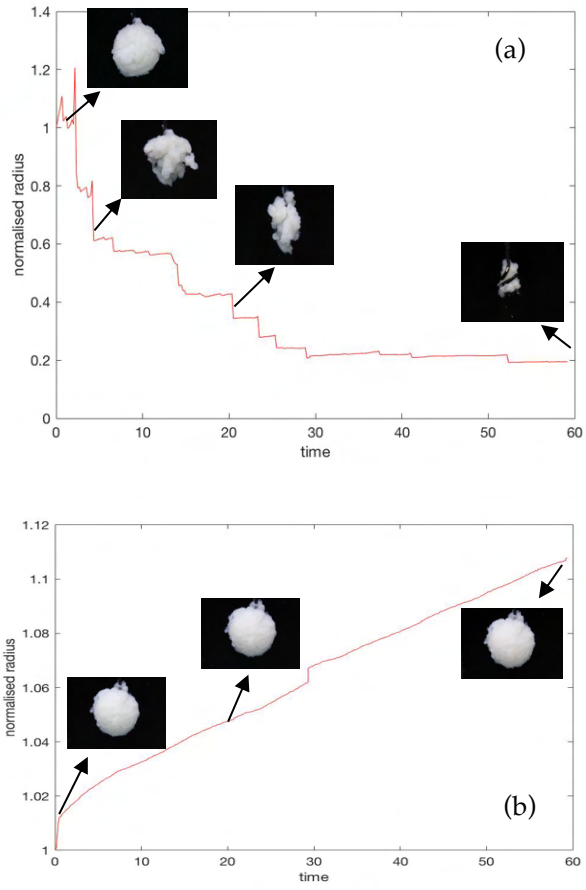


Figure 24. Rice bolus disintegration of cooked rice (a) without and (b) with 1% low acyl gellan gum during simulated gastric environment in phosphate buffer saline pH 2 for 1 hour.

4.3.5. Structural changes during in-vitro small intestinal digestion

To further examine the potential protective effect of LAG on digestion, Figure 25 shows rice grains that have been cooked without (images a) and with (images b) 1% LAG, after immersion to a simulated small intestinal fluid containing α-amylase. In the absence of hydrocolloid, images a1-a4 indicate considerable starch hydrolysis causing removal of small particles and changes in the shape of the rice grain, shown as the grey colour around rice grain image. In contrast, the presence of LAG appears

to limit amylolysis and hold the rice grain intact (images 25b1-25b4). It is reminded that due to sample preparation (see section 4.2.2.6. describing cutting of the rice for this experiment), half cross-sections are shown in Figure 25. In image series b, the left-hand side of the grain is the grain surface, coated with LAG, while the right-hand side is the grain interior, which is not covered with LAG. The side of the grain that is directly exposed to the enzyme shows signs of starch hydrolysis, whereas the side of the grain with the hydrocolloid appears less affected by amylase, further confirming the protective effect of the hydrocolloid on digestion.

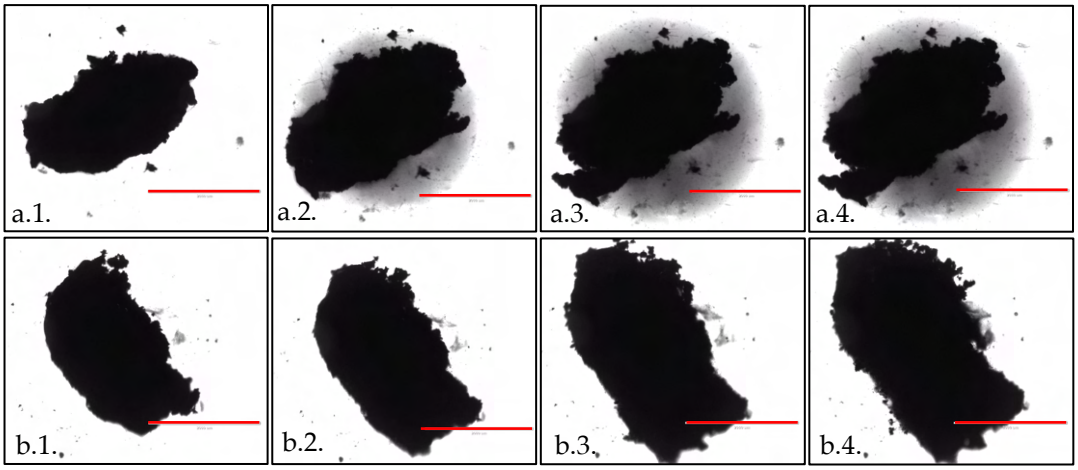


Figure 25. Microscopic images (10x magnification) of rice grain sections (half cross-sections) cooked without (series a) and with (series b) the addition of 1% low acyl gellan gum and further soaked in SIF containing pancreatic alpha-amylase after 0min (1), 10mins (2), 20mins (3) and 30mins (4). Rice grains were stained with Iodine solution (scale bar represents 2mm).

Structural changes during the simulated small intestinal digestion were further observed for the liquid and the solid parts of the chyme. For the liquid part, Figure 26 shows microscopic images before and after digestion, where examples of rice particles are marked in red lines. Both before and after small intestinal digestion, the presence of LAG resulted in larger particles, indicating reduced particle breakdown during

digestion. It appears that LAG protects the rice from the digestive enzymes in all stages of digestion, and one suggested mechanism is, as already mentioned, through the formation of a gel that further strengthens in the acidic environment of the gastric digestion (Bradbeer *et al.*, 2014).

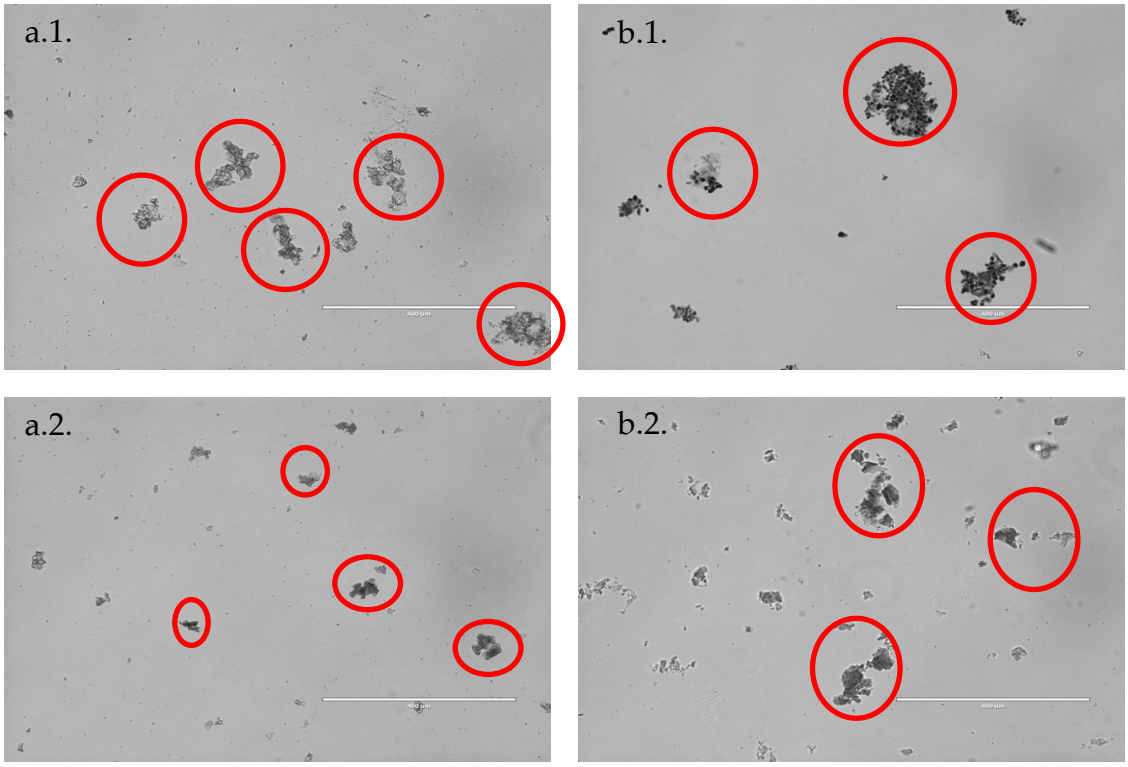


Figure 26. Microscopic images of rice control (a) and rice with 1% gellan gum (b) samples aliquot before (a.1. and b.1) and after intestinal digestion (a.2. and b.2.). Scale bar: 400µm.

Figure 27 shows ESEM images of rice without and with 1% LAG before any digestion steps, as well as after oral, gastric, and small intestinal digestion. This technique has been previously used to visualise the structure of rice grains (Dang and Copeland, 2004). Before any digestion stages, LAG appears to form a smooth layer on the rice grain's surface (indicated in the area enclosed in red lines in Images a1 and b1, and in larger magnification by the difference in the surface smoothness between Images a2 and b2). This layer may be a LAG gel forming on the rice, covering part or

all of its surface. After digestion, the rice with hydrocolloid was less damaged by the digestive enzymes, compared to the rice without LAG. It seems that the presence of 1% LAG on the surface of the rice acts as a barrier that slows down rice digestion, and prevents enzymatic hydrolysis and the associated structural damage caused by the digestive process.

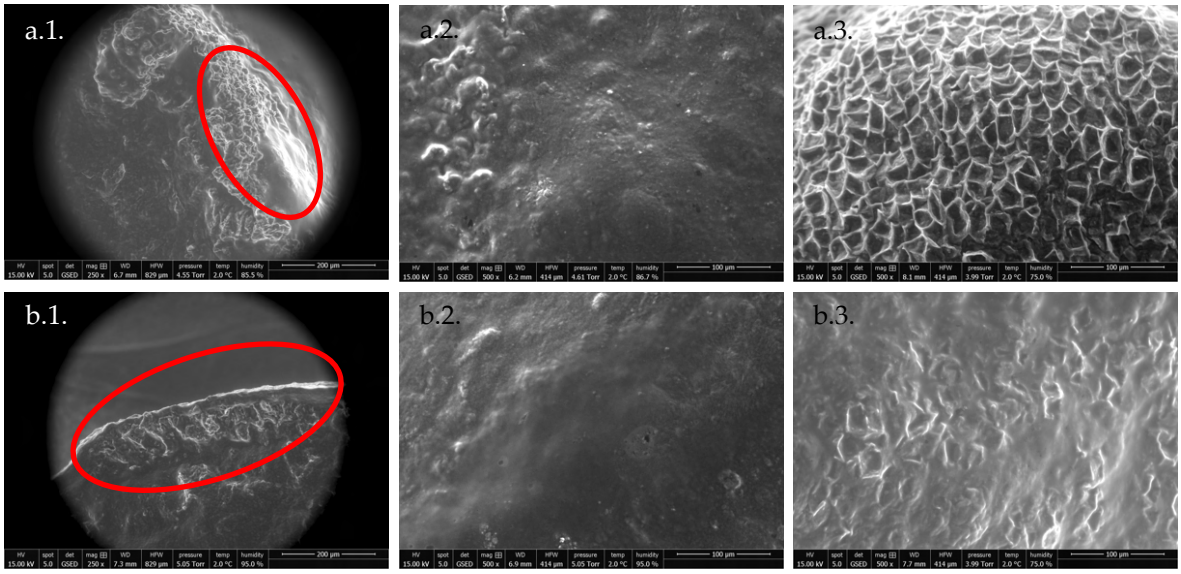


Figure 27. ESEM images of cooked rice (series a) and cooked rice with addition of 1% low acyl gellan gum (series b). Cut sections of cooked rice grains (a.1 and b.1); red circles indicate a thin layer of gel formation on the cooked rice with gellan gum (b.1) compared to intact cooked rice (a.1). Cooked rice surface images before digestion (a.2. and b.2.) and after oral, gastric, and intestinal digestion (a.3. and b.3.).

4.4. Conclusions

This work illustrates the potential of low acyl gellan gum (LAG) to reduce white rice starch digestibility by 30% and estimated glycaemic index (eGI) by 15% when 1% of the hydrocolloid is boiled together with the rice. It is suggested that the mechanism of action may involve structuring of the rice during cooking, as a gel is formed on the surface of the rice grain that obstructs mechanical and enzymatic breakdown of the food. The gel is strengthened at the acidic environment of gastric digestion, further enhancing its protective effect. It would be interesting to investigate binding of the gel to the food and studying whether these findings can extrapolate to other starchy materials, including gluten-free products.

Supplementary materials

1. Time-lapse video of rice bolus disintegration in the simulated gastric environment for 1 hour: https://vimeo.com/501211684
2. Time-lapse video of Rice + 1% low acyl gellan gum bolus disintegration in the simulated gastric environment for 1 hour: https://vimeo.com/501212531
3. Time-lapse video of rice digestion in SIF containing pancreatic amylase for 1 hour: https://vimeo.com/501210094
4. Time-lapse video of rice + 1% low acyl gellan gum digestion in SIF containing pancreatic amylase for 1 hour: https://vimeo.com/501210653

CHAPTER 5

In-vitro Digestion of Rice with Additional Low Acyl Gellan Gum Using Static and Dynamic Models

5.1. Introduction

Human digestion system is an extremely complex system that consist of huge number of biological interactions, such as cells, membranes, genes, organics and inorganics compounds. This complexity makes it difficult to distinguish the associations between singular parts and to investigate how the processes happen. *In-vitro* work which simulate digestive processes outside the body, simplified those complex system then the researcher can give attention to the specific part of components.

Depending on the specific experimental design, *in-vitro* models may mimic all stages of digestion or focus on a specific site (e.g. gastric); they may be mono-compartmental or multi-compartmental, in which the different stages of digestion are simulated in the different compartments; they may further analyse a small amount of material (e.g. in the order of mg) or a large, bite-size substance; or they may be static.

To gain accurate results as close to the its natural process inside the body, a complete *in-vitro* model would simulate all digestive processes as well as its simple to utilise and fast processes. It would likewise be adaptable in its capacities to take into consideration any affectability investigation or varieties in the stomach related conditions wanted to be contemplated. It would likewise be reasonable to work.

In-vitro models usually designed specifically to suit the researcher's main goal. Numerous models have been developed, starting from oral phase, gastric phase small and large intestine phase to static and dynamic models. There are also a number of physiological aspect and environment of digestion might be consolidated *in-vitro* equipments. Those include the temperature, pH and pH inclinations, the protein sorts, fixations and flow rates, addition of digestive liquids, for example, water, electrolytes or mucins, the volumes and flow rates of the foods, retention times, the motility of and mechanical activities applied by the stomach related tract, the dispersion and mass transfer, or the various nutrition types absorption.

Similar with static models, dynamic models may reproduce at least one section of the digestive system. In advance, dynamic models offer the capability of including component of the near-realistic and physiological condition, for example, mechanical forces or peristaltic movements, fluid flow, temperature and pH of surrounding environment, type and concentration of enzymes, and even a similar organelle shapes. The works aimed to study the effect on additional low acyl gellan gum on rice digestion using *in-vitro* food digestion model which is able to mimick peristaltic and contraction in duodenal compared to the simple stirred tank model.

5.2. Materials and Methods

5.2.1. Materials

Jasmine rice (Green Dragon AAA, Thailand) was purchased from local market. Low acyl gellan gum (KELCOGEL® F Gellan Gum) was obtained as a gift from CP Kelco, US. Human Salivary Amylase (HSA), Pepsin, Pancreatic Amylase were obtained from Sigma Aldrich. All chemical and reagent were laboratory analytic grade. Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared following recipe from Infogest in-vitro digestion protocols (Brodkorb *et al.*, 2019) and as describe in Chapter 4.

In this experiment, SSF contained 75 U/ml amylase from human saliva, SGF included 2000 U/ml Pepsin and SIF consisted of 24 U/ml porcine pancreatic amylase in the final concentration. All digestive fluids used were prepared using distilled water the previous day. The enzymes were made freshly on the day of the digestion. All solutions were pre-heated at 37°C in the incubator.

5.2.2. Methods

5.2.2.1. Sample preparation

Jasmine rice was cooked in the mini rice cooker (Cookwork, UK) with and without an additional 1% gellan gum (w/w) using rice and water ratio 1 : 1.5. Cooking time in the rice cooker was 14 minutes, followed with 6 minutes in warm mode. Then cooked rice was stirred using a spoon then the rice was taken out and put into the 500

ml glass beaker covered using aluminium foil. The cooked rice was stored in a 50^0C oven for a maximum of 1 hour until used in the *in-vitro* digestion experiment.

5.2.2.2. Rice structure images

The images of rice structure were captured by DSLR Camera Canon 5D Mark III with MP-E 65 mm macro lens (Canon, Japan). Lightings were used on each side left and right from 2 studio LED light (Aputure, Amaran AL-528W lighting with 90% of light intensity). Sample images of rice control and rice with additional 1% low acyl gellan gum were taken on following condition, freshly cooked, after simulated oral mastication, after 30 minutes of gastric digestion and after 180 minutes of simulated small intestine phase. Then, rice structure images after 3 hours of small intestine digestion were compared between output from STR and DDM (with and without contraction 2cpm). Particle area of cooked rice was measured by image processing using ImageJ software.

5.2.2.3. ESEM images of rice

Some samples of rice grain were carefully taken during small intestine digestion on 20 minutes and 120 minutes as representing rapid digestibility starch (RDS) and slow digestibility starch (SDS) sample, respectively. The samples were then soaked in 0.3 M Na_2CO_3 solution for 1 minutes to stop enzyme reaction. Rice sample were stored in 2°C fridge for ESEM analysis on the next day.

Environmental Scanning Electron Microscope (ESEM) images were captured using FEI Quanta 650 (The Thermo Scientific, UK). Rice grain samples before and after digestion process were cut in cross section and lengthwise section with the thickness of 1 mm using sterile surgical blade. The cut section the placed on the sample stub and

glued with carbon cement. One ESEM session was analysing 4 samples of 2 cut section for rice inner structure measurement and 2 lenghwise section for capturing surface structure. The ESEM analysis was conducted under condition of power 15.00kV, 500x magnification, 4.25-5.05 Torr pressure, temperature 2^0C and humidity 80-95%. A large number of images have been taken in each experiment and representable images of each condition are presented here for structure comparison.

5.2.2.4. Simulated oral phase digestion

Cooked rice (20g) with or without addition gellan gum was placed in the stainless container of mini coffee grinder (Cookwork, UK). Then 20ml of SSF containing 75 U of α-amylase from human saliva (A1031-5KU, Sigma, UK) was added to the grinder. Oral processing was mimicked by mixing the cooked rice with SSF in the coffee grinder for 5 seconds with pulse in every second.

5.2.2.5. Simulated gastric phase digestion

Bolus from simulated oral processing was collected and transferred to 250 ml jacketed vial, as shown in Figure 28. Temperature of the chamber was maintained at 37^0C by channelling water from water bath. The jacketed vial was put on the 37^0C hot plate stirrer. The gastric phase digestion was started after the addition of 40 ml of SGF containing 2000 U/ml of pepsin (P700, Sigma, activity: 695 U/mg) in to the bolus. The gastric processing was conducted for 30 minutes under gentle mixing by magnetic stirrer at 75 rpm. The chyme sample from the gastric digestion will be transfered to the small intestinal phase conducted in the stirrer tank reactor (STR) or dynamic duodenal model (DDM).

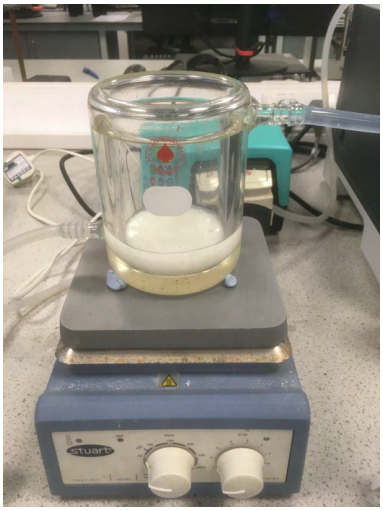


Figure 28. Simulated gastric digestion using jacketed vessel with continuous mixing

5.2.2.6. Food digestion models

On this experiment, 2 custom made food digestion models developed were compared. The Stirred Reactor Tank (STR) consists of a semi-permeable cylindrical container placed inside a jacketed vessel as shown in Figure 29. In this configuration, the food sample and simulated digestive liquid were processed inside the cylindrical tank and the continuous gentle mixing at 75 rpm was achieved by using overhead stirrer (Hei-Torque Precision 100, Heidolph, Germany). Temperature was kept constant at 37°C by flowing water from a water bath into the jacketed vessel. The area outside the permeable tank, representing the recipient medium of nutrient absorption, was initially filled with distilled water with continuous mixing using magnetic stirrer.

As mentioned above in Chapter 3 developing methods, Dynamic duodenum model (DDM) was developed to mimicking nutrient absorption in small intestine with the presence of segmentation and/or peristaltic force. The appearance of DDM model shows in Figure 30. The inner tube is a semi-permeable membrane representing the small intestine, which is filled with chyme and digestive liquids. The outer layer of the

membrane, the annular area between the semi-permeable dialysis membrane and the impermeable silicone tube that surrounds it, is the recipient area, where small molecules that are generated through hydrolysis of macromolecules are collected and measured. In this experiment, DDM was set with or without segmentation movement 2 cpm (contraction per minute).

Chyme from oral and gastric phase processing was transferred into cylindrical tank and processed as small intestinal phase for 180 minutes. 20µl of samples were pipetted one each time point (0, 5, 10, 15, 20, 30, 45, 60, 90, 120, and 180 minutes).

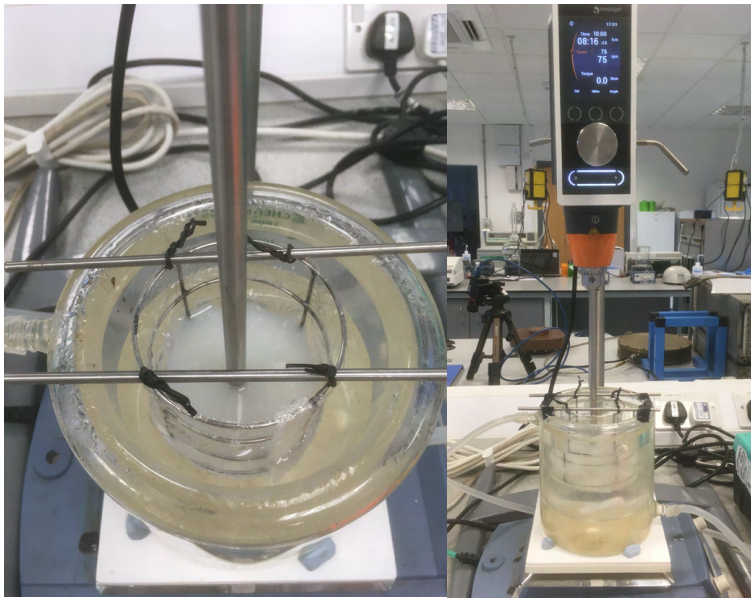


Figure 29. Simulated intestinal digestion using Stirred Reactor Tank (STR) for food digestion experiment

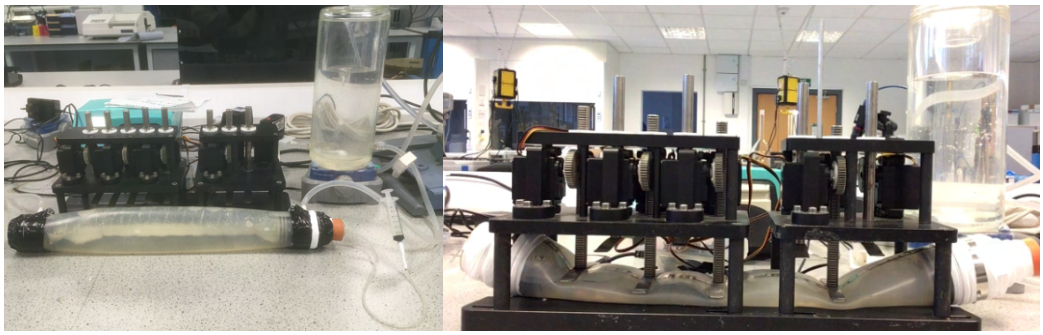


Figure 30. Simulated small intestinal digestion using Dynamic Duodenal Models (DDM) without contraction force (left) and with contraction force 2 cpm (right)

5.2.2.7. Models fitting

The following kinetic models were used in this experiments for comparison:

1. Michaelis-Menten model was use for the starch hydrolysis and it were estimated from the reaction advancement. As assumption, the concentration starch as substrate is significantly higher than the concentration of the enzyme (Fonseca, 2012). The equation are as following.

   $$\frac{dS}{dt} = \frac{V_{max}\, S}{(K_m + S)} \quad (6)$$

   $$K_m \ln\left(\frac{S_o}{S}\right) + S_o - S = V_{max}\, t \quad (7)$$

   where S is the substrate concentration, So is initial substrate concentration, Vmax is the maximal rate and Km is Michaelis half saturation constant.

2. First order kinetic is simple and has been used in the food studies, such as starch hydrolysis and glycaemic index (Goni, Garcia-Alonso and Saura-Calixto, 1997). Mahasukhonthachat, Sopade and Gidley (2010) used first order kinetic on starch digestion of sorghum as effected by particle size. The model was calculated as below equation.

   $$Y = Y_0 + (Plateau - Y_0) \times (1 - \exp(-Kx)) \quad (8)$$

3. Two phase association kinetics:

   $$SpanFast = (Plateau - Y_0) \times PercentFast \times 0.01 \quad (9)$$

   $$SpanSlow = (Plateau - Y_0) \times (100 - PercentFast) \times 0.01 \quad (10)$$

   $$Y = Y_0 + SpanFast \times (1 - \exp(-KFast \times X)) + SpanSlow(1 - \exp(-KSlow \times X)) \quad (11)$$

where Y_0 is the value of Y when X (time) is zero. $Plateau$ is the value of Y at infinite intervals, expressed and defined in the same units as Y. $KFast$ and $KSlow$ are two rate constant, expressed in reciprocal X-axis time units. If X is in minute, then K is inverse minute (GraphPad Prims 8 tutorial, 2019).

All the kinetic models calculation and the graph developing were made using Graphpad Prism 8 software. The numbers taken from at least duplicate experiments data and presented mean data with standard deviation.

5.2.2.8. Mass Transfer Coefficient

Mass transfer coefficient of sugar reduction (maltose) during rice digestion was calculated based on previous work (Gouseti *et al.*, 2014; Jaime-Fonseca *et al.*, 2016) using these equation:

$$A = \pi . D . L \quad (12)$$

$$M_T = \frac{mol_{maltose}}{A.t} \quad (13)$$

$$K_{overall} = \frac{M}{\Delta C} \quad (14)$$

The STR experiment consists of a cylindrical metal frame with a diameter of 75mm, this is covered with a permeable membrane, the membrane is made up from 3.5kDa dialysis tubing (snakeskin dialysis tubing, Thermo Scientific), with the bottom of the cylinder fixed with non permeable material, giving A is surface area for absorption (m^2), D is the diameter of the frame (m), and h is the height of the liquid in the STR compartment. The DDM experiment made of outer silicone tube and inner tube of permeable membrane with diameter of 35 mm and length of 370 mm. The equation

will define for D is the diameter of dialysis membrane (m) and L is length of DDM tubing (m). ΔC is the concentration difference (mol m^{-3}) between the two sides of the membrane. $mol_{maltose}$ is the maltose in the recipient side (mol) and M_T the total molar flux (mol $m^{-2}s^{-1}$).

5.2.2.9. Rice sensory evaluation

The rice sensory test were performed according to Shirani and Ganesharanee (2009); Zhong *et al.*, (2014) with some modification. Rice sample was prepared in the food hall research facility and the sensory analysis was performed in a three-booth sensory evaluation laboratory. Cooked rice samples were prepared as follow. A 200 g of rice (Jasmine, Fragrant Rice, Green Dragon Brand AAA, Thom Mali, Thailand) with or without addition 1% low acyl gellan gum powder were added to the rice cooker pan. After mixing with a spoon to disperse the gellan gum powder, 300 ml of tap water was added. Then the rice grain was cooked in the rice cooker (Elgento 1.5 L, RKW Ltd, UK). In the average, cook-mode went for 14 minutes and automatically switch to warm-mode. After 20 minutes in the rice cooker, cooked rice was transferred to aluminum bowl with lid. The samples then kept in the electronic oven with temperature at 50^0C to keep warm until served to the panelist.

Twenty panellists familiar with rice as a staple food, 10 Asian and 11 European ethnic, were research staff and graduate students of Division of Food Science and Technology, University of Nottingham. The sensory test of cooked rice was divided into 2 sets, the differentiation test and organoleptic acceptance test. The samples were marked with 3-coded number and presented in the randomized order. The panellists were provided with a cup of water and asked to rinse the mouth then drink between

the samples. Figure 31 shows the sensory test conducted in sensory laboratory (a and b), samples presentation for triangle test (c) and organoleptic preference test (d).

In the first set of test, the triangle sensory test as describe in Lawless and Heymann (2010) was conducted to evaluate the different structure between cooked rice control and rice with an additional 1% gellan gum. Panellists were provided with three samples of cooked rice two of which are identical. The panellists were asked to select the sample perceived as different.

Statistical analysis of triangle test was using the normal distribution and Z-test on proportion with specified values of the normal deviate (z). The following formula was used to calculate z-value (Stone and Sidel (1978) in Lawless and Heymann, 2010).

$$z = \frac{[P_{obs} - P_{change}] - \frac{1}{2N}}{\sqrt{pq/N}} \qquad (15)$$

where,

$P_{obs} = X/N$

$P_{chance} = 1/3$

X = number of correct judgement

n = total number of responses

p = probability of correct decision by chance

$q = 1 - p$

The critical Z- value for a one-tailed test at alpha (α) of 5% is 1.645.

The second set of test was the organoleptic preference of two rice samples. Two sample of rice with and without additional gellan gum were presented to the panellists. Written instructions were given. The panellists were instructed to evaluate the preferences of the products on the basis of their taste, texture, colour and overall acceptability, using a nine-point hedonic scale (1 = dislike extremely to 9 = like

extremely). In addition, using the same samples, panellist were been asked to evaluate the rice hardness and stickiness with the scale of 1=very soft, 5=very hard for its hardness and 1=very dry, 5=very sticky for stickiness parameter. Statistical significance determined by t-test with alpha = 0.05 using Prism 8 GraphPad software.

Figure 31. Sensory evaluation of cooked rice with and without addition low acyl gellan gum in the sensory laboratory (a and b). Panellist were presented with set of sample for triangle test (c) and organoleptic preference test (d)

5.2.2.10. Preliminary study of MRI analysis of rice digestion

In a pilot study a healthy volunteer gave informed written consent (Institutional Review Board Ethics approval 470-2001). According to the protocol to be followed in the in-vivo MRI studies, the volunteers were to fast overnight before the MRI study. In the morning they were fed a portion of cooked jasmine rice equivalent to 232 kcal energy served with a 300 ML drink of water. The rice was cooked with and without 3% gellan gum. Images of the rice meal in the stomach were then taken at intervals on a GE 1.5T HDx MRI scanner using a range of imaging sequences. Each image set was acquired under a short breath-hold to minimise respiratory motion.

5.3. Results and Discussion

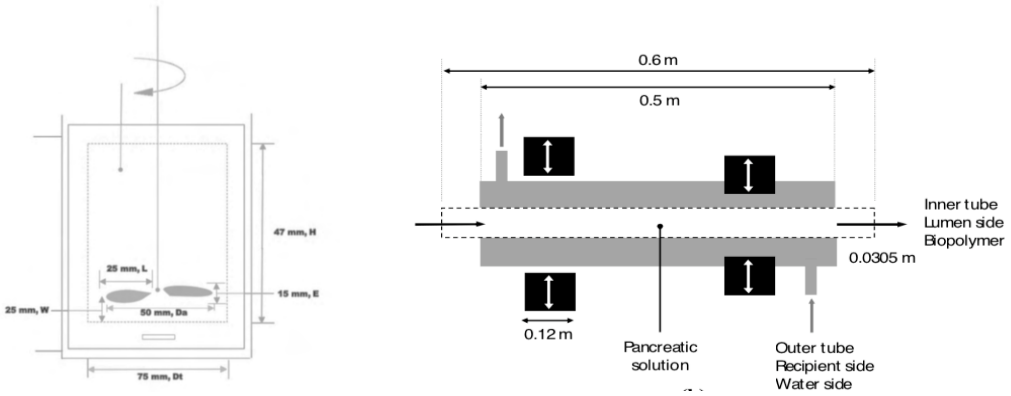
5.3.1. In-vitro digestion models

The stirred tank reactor (STR) and dynamic duodenum model (DDM) were designed to study mixing process and mass transfer on the in-vitro food digestion. The previous STR model was designed by Latty (2019) for model fluids and bread mass transfer measurement. The present DDM model was developed based on earlier versions of small intestine models, also developed in this lab (Fonseca, 2012; Gouseti *et al.*, 2014; Latty, 2019). The diagrams of the two models, STR and DDM, are shown in Figure 32 and the working mechanisms are as explained in Chapter 3.

The STR models offered a simple approach to measure mass transfer from permeable walled cylinder tank to liquid solution outside the membrane. Sample collection can be taken easily from both sides, inside the reactor tank and outside area representing recipient media. It is a flexible and easy to operate model. However, the STR model has a limitation compared with others in-vitro digestions model. Although there is a mixing element, the cylindrical shape and overhead stirrer mixing process will not be able to represent an actual process in the human small intestine.

The DDM designed to mimicking the actual process occurred in the duodenum section of small intestine. It allows mechanical compression that can be set to run contraction and peristaltic movement, similar to that occurring in the human gut system. The inner tube was using semipermeable dialysis membrane, which allows small, hydrolysed nutritional molecules to pass through the membrane and transfer to the outer layer that initially consisted of distilled water. The models expected to

evaluate bioaccessibility of nutrients at dynamic conditions that account for mixing and mass transfer in the gut.



(STR) **(DDM)**

Figure 32. Stirred tank reactor (STR) and Dynamic duodenal model (DDM) diagram, taken from Latty (2019)

5.3.2. Rice structures

Structure of cooked rice with and without additional 1% low acyl gellan gum before the digestion process, after simulated oral, gastric and intestinal phase using STR, DDM and DDM 2 cpm are shown in Table 4. Cooked rice with additional 1% low acyl gellan gum appeared to have bigger size than cooked rice control. Particle area of the cooked rice with addition 1% low acyl gellan gum was 23.5 ± 0.5 mm^2 compared to rice control that was 21.7 ± 0.3 mm^2. This phenomenon probably happened due to the presence of an additional gel layer which appeared on the cooked rice with 1% gellan gum. The gel layer was confirmed by ESEM images as shows in the Figure 20 and 27 on Chapter 4.

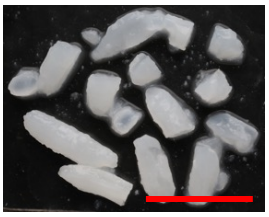
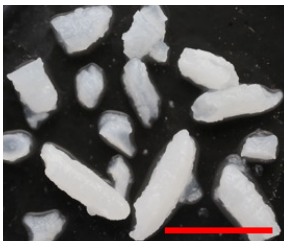
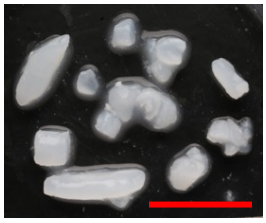
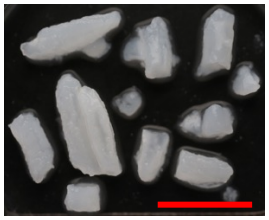
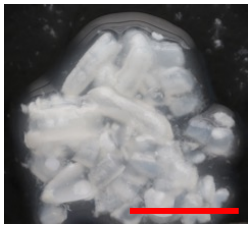
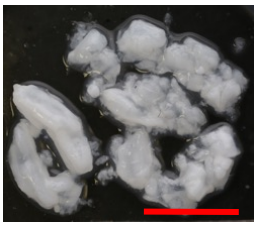
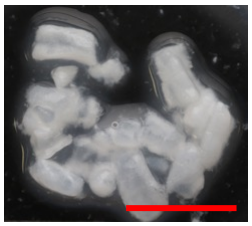
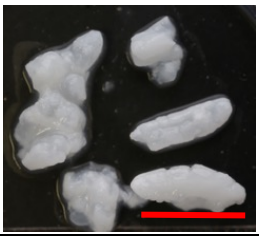
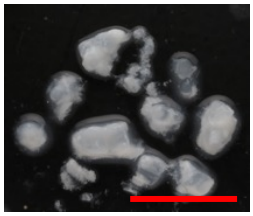
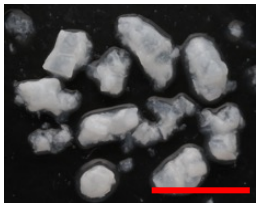
After the simulated oral processing, both rice samples showed similar structures with various cutting size, from intact grain size, to half, quarter, or smaller

fractions. However, during the gastric digestion, the rice with gellan gum was less digested than the control. Some grain bind together and hold similar shape as a bolus after oral processing. The low acyl gellan gum formed a harder gel under acid environment (CPKelco, 2013). In a previous work, the low acyl gellan gum prepared at natural pH had the highest gel strength when it was exposed to the low acid solution of pH 2 for 24 hours and the strength decreased when the system was exposed in acid solutions higher than pH 3 (Bradbeer *et al.*, 2014; Cassanelli *et al.*, 2018). The mixing process during gastric digestion seems give less effect to particle breakdown of rice with low acyl gellan gum.

Comparing rice structure after 3 hours of *in-vitro* small intestine digestion showed that using DDM with contraction mode (2 cpm) gave more particle size reduction. On the other hand, DDM static gave less digested rice particle. Contraction force played a role on the mixing process during small intestinal digestion and enhanced food digestibility, which has been reported before (Gouseti *et al.*, 2019).

Between two type of sample, rice particle with addition gellan gum appeared to be less broken down compared to rice control, especially when processed using DDM static. The structure images shown rice control sample became more transparent and swelled than rice with gellan gum. It can be seen that contraction in DDM enhanced rice digestibility for both type of rice. Rice control shown more digested in the DDM with contraction (2 cpm).

Table 4. Rice structures during in-vitro digestion using STR, DDM static and DDM 2 cpm

	Rice Control	Rice + 1% low acyl gellan gum
Cooked		
After simulated oral processing		
After simulated gastric processing		
After 3 hours of simulated intestinal processing using STR		
After 3 hours of simulated intestinal processing using DDM static		
After 3 hours of simulated intestinal processing using DDM 2 cpm		

In order to analyse the effect of addition of low acyl gellan gum on the rice structure, ESEM examination has been conducted. The ESEM allows samples to be imaged at high resolution with simple specimen preparation. The ESEM images of rice surface before and after the digestion process are shown in Table 5. Before the digestion process, cooked rice with additional 1% low acyl gellan gum had a smoother outer layer compared to rice control. This appearance might be due to the formation of gel layer covering rice surface. After fully hydrating, low acyl gellan gum immediately forms gels in the temperature range of 30-50^{0}C (CP Kelco US, 2007).

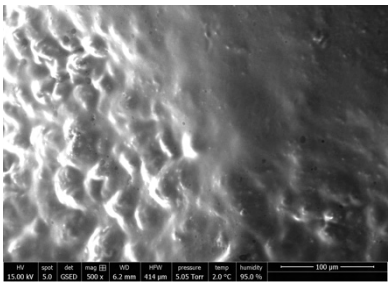
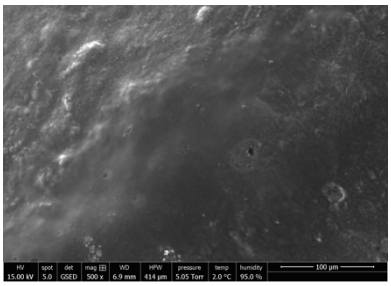
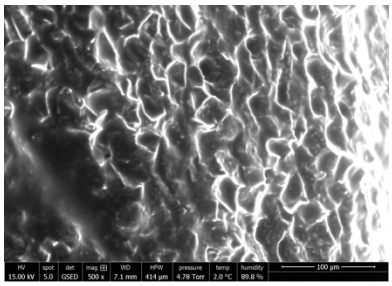
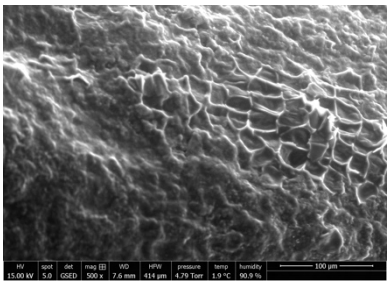
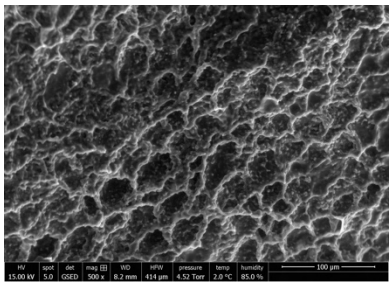
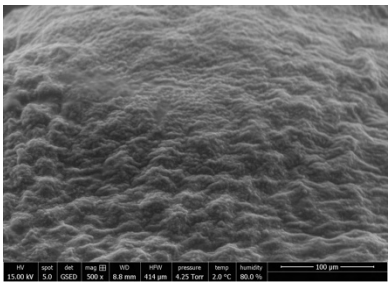
Images of the rice surface after 20 minutes of simulated small intestinal digestion show that rice control has more damaged surface than rice with low acyl gellan gum. It appears that the intestinal liquid and amylase enzyme penetrated easier the rice control grain than the rice with gellan gum. The rice grain with additional low acyl gellan gum was probably protected by a gel layer formed as the material went through the acidic gastric phase environment. Low acyl gellan gum will form firm and brittle gel on acidic solution (CP Kelco US, 2007). This might prevent the enzyme to penetrate the rice surface.

During 2 hours of digestion, further hydration happened, rice surface continued to swell and disintegrate. Rice control had a massive destruction while absorbing the intestinal liquid. On the other hand, rice with low acyl gellan gum appeared to be less eradicated. The images could indicate that slow digestible starch (SDS) of rice with addition low acyl gellan gum was higher than rice control.

Taking high resolution images of specimen with high moisture content is possible using ESEM. The rice sample can be examined with less speciment preparation. One study on capturing organisational structure of uncooked dan preboiled rice grain has been successfully conducted using ESEM (Dang and

Copeland, 2004). Our aim to study the starch structures after digestion was not possible to be done. The high moisture content and the gel-like structure of digested sample were the limitation to have a required speciment.

Table 5. ESEM images of rice surface microstructure

	Rice Control	Rice + 1% low acyl gellan gum
Cooked rice		
After 20 minutes of simulated intestinal processing		
After 2 hours of simulated intestinal processing		

5.3.3. Rice digestion in STR and DDM

Figure 33 shows starch hydrolysis (%) as a result of in-vitro rice digestion using a static digestion method based on the Infogest protocol, the stirred tank reactor (STR) and the dynamic duodenum model (DDM) with and without contraction force (2 cpm). Using all methods of in-vitro digestion in this study show that samples of rice with additional 1% gellan gum have slower digestion rate compared to rice control. DDM static gives the least hydrolysis rate, and static Infogest methods induce the fastest starch hydrolysis, as expected. The highest percentage of hydrolysed starch was produced by rice control digestion using DDM 2 cpm. While the addition of gellan gum could reduce rice digestibility by up to approximately 20%.

Comparing of in-vitro digestion models used in this experiment, in average STR gave the highest digestibility rate and DDM without contraction was the lowest. STR used magnetic stirrer with continuous agitation that gives an more effective mixing process leading to induce enzymatic starch hydrolysis. Overall, the addition 1% gellan gum slowed rice digestibility. Our previous study on rice digestion using Infogest's static methods as shown in Chapter 4 have the similar phenomena.

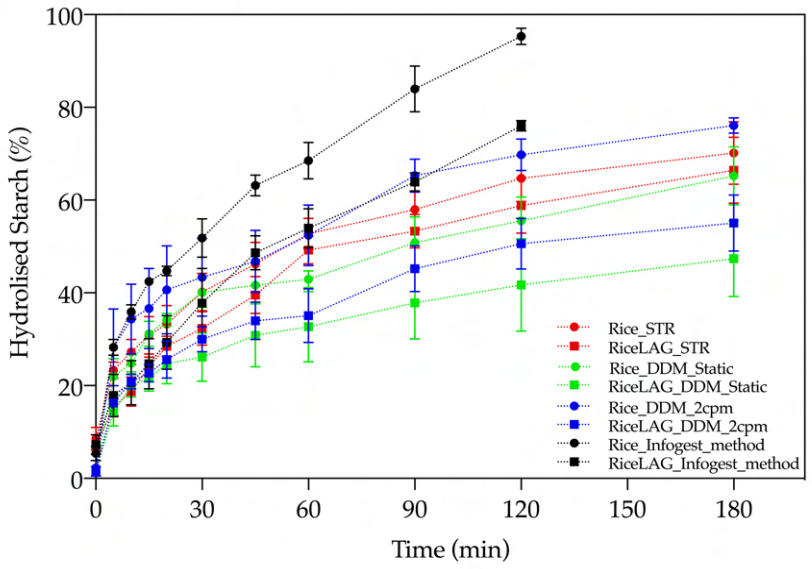


Figure 33. Starch hydrolysis rate of rice with and without addition 1% low acyl gellan gum (LAG) on in-vitro digestion using stirred reactor tank (STR) and Dynamic Duodenal Model (DDM), compared to static Infogest methods. Values are means of triplicate ± the standard error of the mean (SEM).

5.3.4. Fitting the curve with kinetic models

In the Figure 34, intestinal starch hydrolysis rate of rice and rice with gellan gym using the STR model and the DDM model without contractions and at 2 cpm are shown and compared with three kinetic models, which were fitted to the experimental data. The three models: Michaelis-Menten enzyme kinetic (b, f), first-order kinetic (c, g) and exponential of two phases model (d, h) are presented.

Comparison of Figures 34 (a) and (e) shows that in all cases, addition of low acyl gellan gum overall resulted in reduced starch hydrolysis rate. This is evident in particular in the first 30 minutes of intestinal digestion, the region of the curve before plateau occurs. The reduced starch hydrolysis rate further resulted in lower "final"

starch hydrolysis values, the values of the plateau, in the presence of gellan gum compared to those of the rice control. These findings are in agreement with the findings of Chapter 4.

The digestion rate after 30 minutes began to slow. Based on the kinetic models and experiment results, the digestion process was starting to reach plateau after 90 minutes. The addition of gellan gum appeared to slowing the digestion rate. Overall, rice with additional 1% gellan gum inhibits the digestion rate on all in-vitro digestion models.

Comparing the three digestion envioronments (STR, DDM without contractions and DDM at 2 cpm), the static DDM showed the slowest digestion rate for both rice and rice with gellan gum. The interesting finding was the use of STR which gave highest digestion rate on rice with gellan gum compares to DDM 2 cpm. On the other hand rice control samples were digested faster in the DDM 2 cpm than using STR. For the rice samples, the increased mixing of the STR, compared to that in the DDM at 2 cpm (see for example the mass transfer coefficients in the next section), seems to have promoted higher digestion rates. In the case of rice with gellan gum, it appears that the weak low acyl gellan gum gel is unable to hold its structure during the continuous mixing of the magnetic stirrer. However, when using the DDM model at 2 cpm contractions, the low acyl gellan gum gel appeared strong enough to hold its structure.

The Root Mean Standard Deviation (RMSD) values of percentage digested starch between each kinetic models and samples experiment are presented in Table 6. In aggrement with the experimental data, all models kinetic has a similar results with average values are 8%, 9% and 7% for Micahelis-Menten, First Order and Two-phase model, recpectively. Two-phase model predicts the best agreement in all experiment

graphs. In addition, all models fit the best on the RiceLAG in STR experiment, while the least is on Rice on DDM 2 cpm data.

Table 6. RMSD value of digested starch (%) between the samples and three kinetic models

Models	RMSD					
	Rice STR	Rice DDM_Static	Rice DDM_2cpm	RiceLAG STR	RiceLAG DDM_Static	RiceLAG DDM_2cpm
Michaelis-Menten	7%	7%	11%	6%	9%	7%
First Order	9%	8%	12%	7%	10%	7%
Two-phase	6%	5%	10%	6%	9%	6%

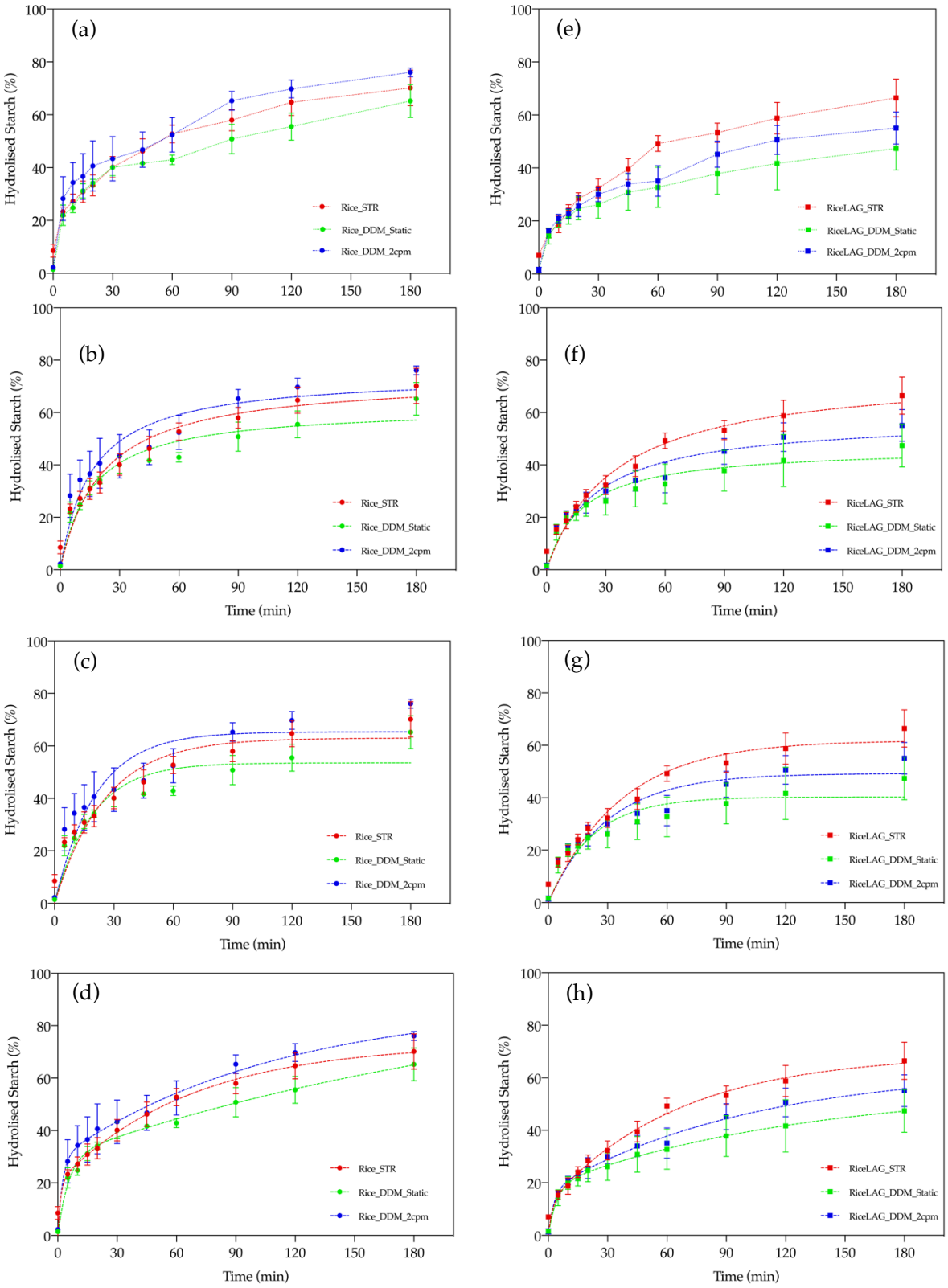


Figure 34. Diagram of Rice (a-d) and Rice with additional 1% low acyl gellan gum (e-h) as results of STR, DDM static and DDM 2cpm methods (a, e), curve line fitted by Michaelis-Menten enzyme kinetics (b, f), first-order kinetic (c, g) and exponential association of two phases (d, h). Values are means of triplicate ± the standard error of the mean (SEM).

5.3.5. Effect of additional 1% low acyl gellan gum on in-vitro rice digestion

The effect of additional 1% low acyl gellan gum to the rice digestibility shows in the Figure 35. Graphs are presented as a percentage of hydrolysed starch versus digestion time (minutes) fitting with a first order kinetic model. As one can see in the red line graph, the rice digestion between the two samples in the STR gave only a slightly different kinetic rate, with predicted plateu for Rice_STR at 63% and RiceLAG_STR at 62%. This result shows that the mixing process in the STR model was able to induce digestion of rice with low acyl gellan gum at a rate similar to that of the rice control.

The green line graph shows digestion rate of rice samples in the DDM model without applying any contractions. The results exhibited that enzymatic hydrolysis process still occurred even without mixing. The first order kinetic model predicts that hydrolysed starch of rice control will reach a plateau of 53% while rice gellan gum on 40%. The hydrolysis rate was lower compared to digestion model STR and DDM with contraction and the addition of gellan gum gave the lowest

Similar on the STR and DDM static figures, the blue line graph shows that low acyl gellan gum obstructed rice digestibility when digestion occurred in the DDM model with 2 cpm applied contraction rate. The digested plateu of rice with gellan gum will be reached at 49% compared to 65% of the rice control. Overall, the addition of gellan gum to the cooked rice reduced its digestibility on a DDM *in-vitro* digestion model.

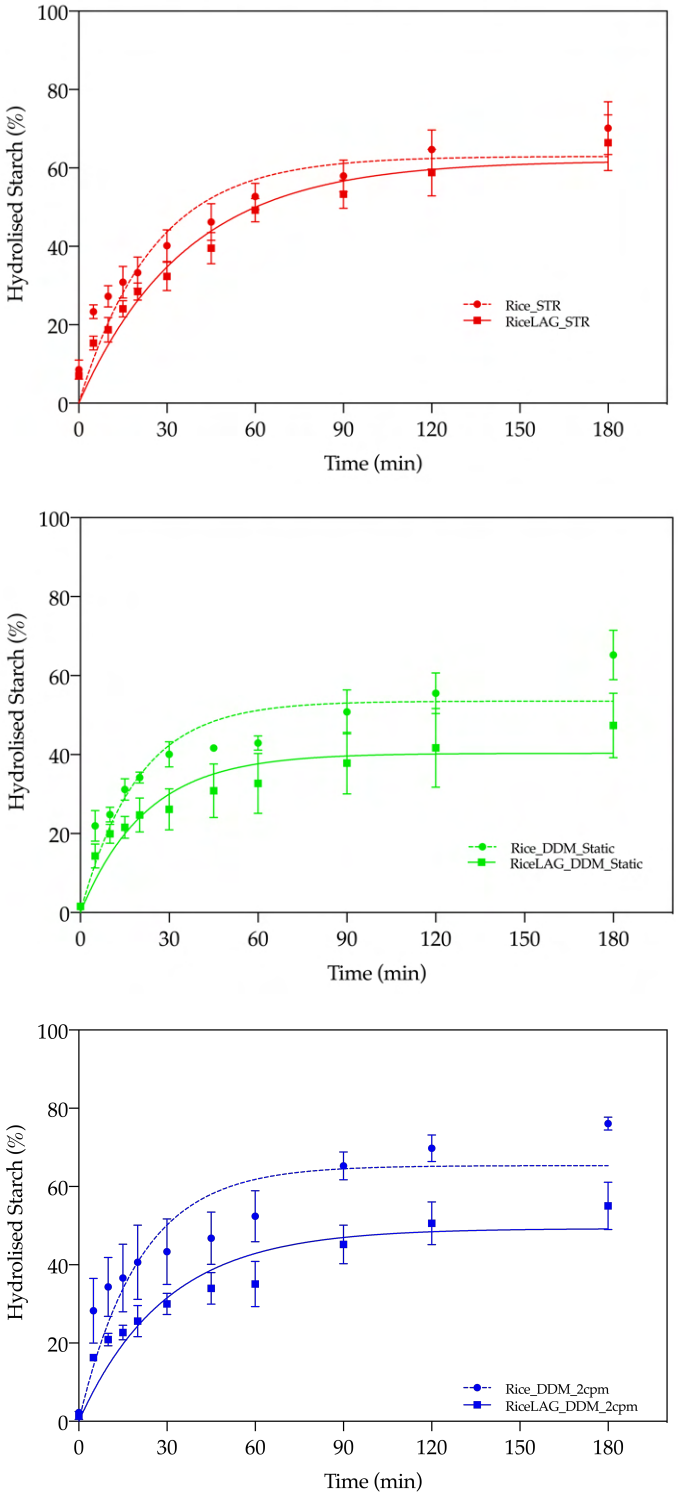


Figure 35. Rice digestibility as results of STR, DDM static and DDM 2cpm models, curve line fitted by first order kinetics (using data table of percentage of hydrolysed starch vs time). Values are means of triplicate ± the standard error of the mean (SEM).

5.3.6. The effect of pressing mode of DDM on rice digestion

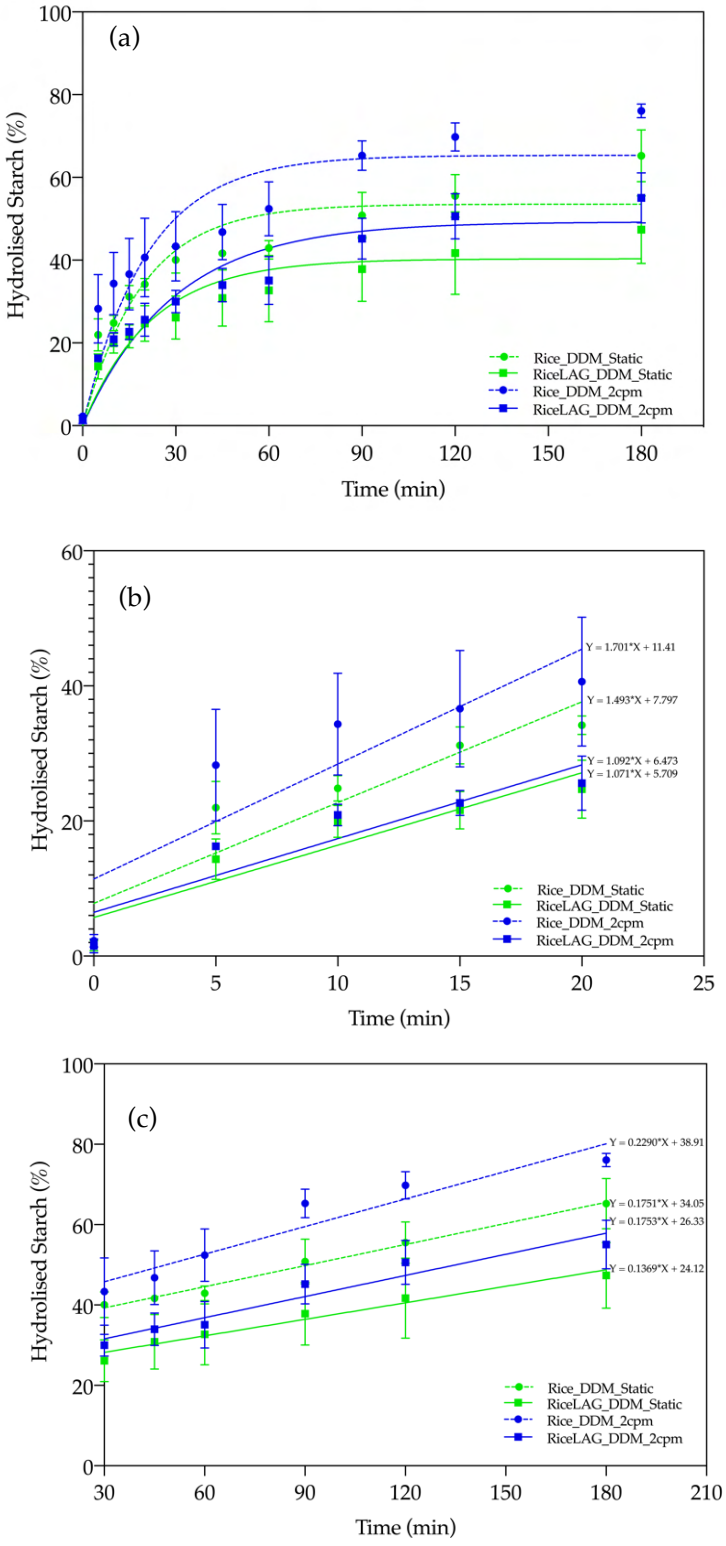


Figure 36. Comparing rice digestibility using DDM, curves fitted by first-order kinetics (a); linier regression for Rapid Digestible Starch/RDS or 0-20 minutes of digestion (b) and Slow Digestible Starch/SDS or 30-180 minutes of digestion (c). Values are means of triplicate ± the standard error of the mean (SEM).

Figure 36 compares the starch hydrolysis rate of the rice and the rice with gellan gum samples when digestion was carried out using the DDM model with and without the use of contraction mode. The contraction of 2 cpm enhanced the hydrolysed starch plateu by 22% which changing from 54% to 65% and from 40% to 49% on rice control and rice with addition gellan gum samples, respectively. Rice with additional LAG could inhibit starch hydrolysis by 30% on contraction mode and 20% on static mode. In the early digestion phase (0-20 minutes) known as rapid digestible starch (RDS), the use of contraction mode show different trend to digestibility of rice samples with rice in the DDM 2 cpm show the fastest digestion rate. Rice sample digestion increased with the use of contraction mode.

In the slow digestible starch phase (i.e. 30-120 minutes of intestinal digestion), the addition of LAG inhibited the rice digestibility on both modes, static and with application of 2 cpm contractions. The use of 2 cpm mode was induce the rice digestibility for 29-30%.

5.3.7. Mass transfer of reducing sugar

Figures 37 and 38 show the released reducing sugars sampled in the inside and the outside of the dialysis membrane tube, respectively, for the experiments conducted with the STR model and the DDM model without and with the application of 2 cpm contractions. As a result of starch hydrolysis by pancreatic amylase, maltose concentration inside the dialysis membrane increased as the intensity of mixing in the luminal area increased. As a result, the higher mixing applied by the magnetic stirrer in the STR model produced the highest maltose concentration, compared to that when mixing was performed by 2 contractions per minute (cpm). In the inside area of the

dialysis membrane, non-contracted DDM showed the least maltose concentration. Similar trends can be found in dynamic in-vitro digestion model of bread (Gouseti *et al.*, 2019), and glucose solution (Gouseti *et al.*, 2014; Jaime-Fonseca *et al.*, 2016), where application of contractions resulted in increased mass transfer rates and higher starch hydrolysis rates.

Maltose concentration in the area outside the dialysis membrane (recipient area) showed different trends compared to the inside area. The DDM without application of any contractions (static) has the highest maltose concentration, meanwhile STR shows the least maltose concentration. DDM has more surface area than STR. DDM static gives higher maltose concentration in the recipient area that DDM with 2 cpm. This phenomenon affected by the flowability of water in the recipient area. The contraction suppressed the digestion tube which retard the flow of recipient area water. On the other hand, DDM static model produced a swift flow of recipient water. It can maintain the gap between outer tube and dialysis membrane.

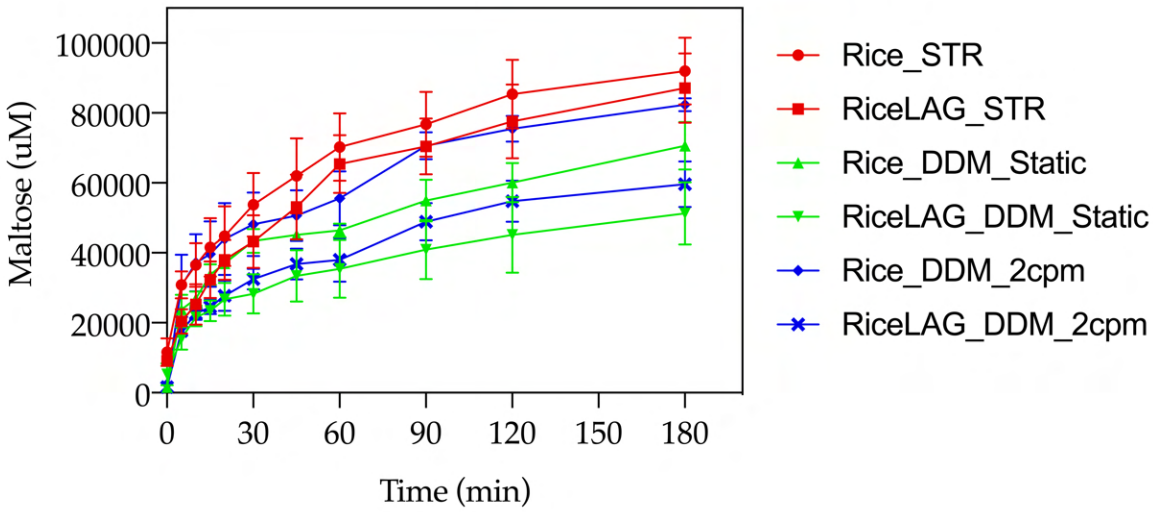


Figure 37. Reduction sugar inside the dialysis membrane. Values are means of triplicate ± the standard error of the mean (SEM).

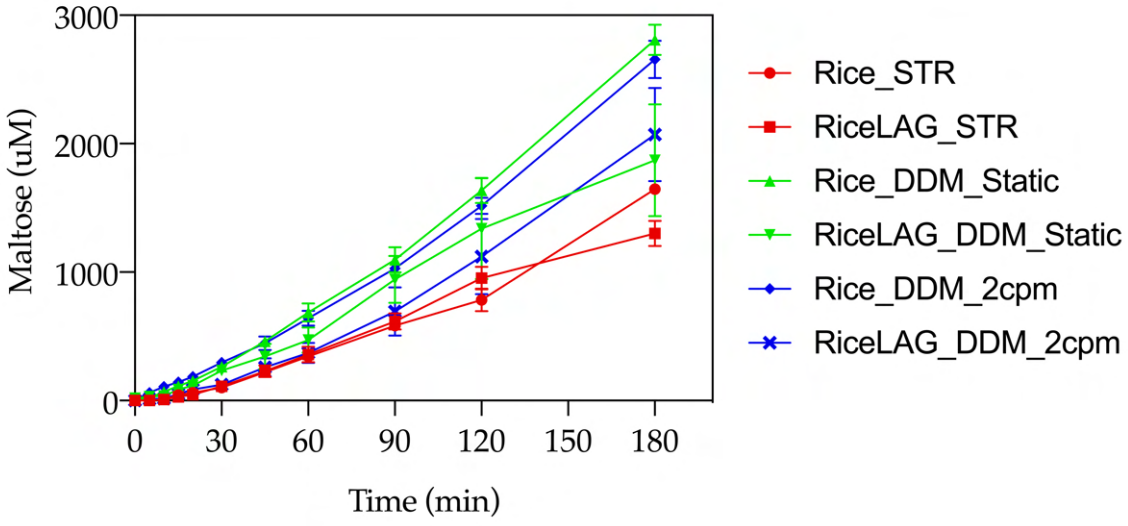


Figure 38. Reduction sugar concentration in the recipient area (outside dialysis membrane). Values are means of triplicate ± the standard error of the mean (SEM).

Figure 39 shows the mass transfer coefficient of maltose from inside dialysis membrane to the recipient area. As a results of contraction, DDM with 2cpm gave lower mass transfer coefficient compared to DDM static. STR shows highest mass transfer coefficent by its continues agitation. From the results one can see that the rice sample was broken down faster in the DDM with contraction. However, mass transfer coefficient acted differently. It's maltose substance transferred slower than DDM static. This finding may be because during contraction, the tube was pressed regularly impeded flowability of recipient liquid as seen in the Figure 28, the comparation of DDM mode between static and 2 cpm contractions.

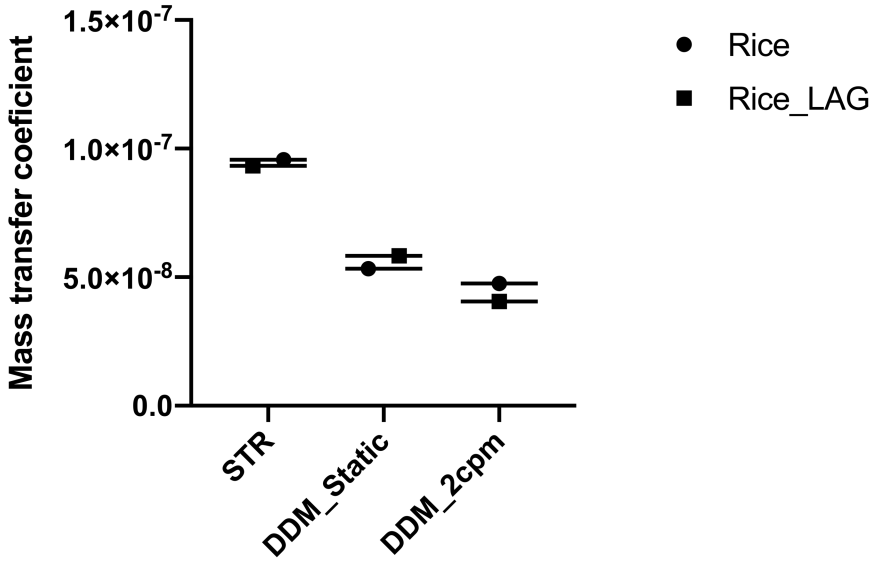


Figure 39. Mass transfer coefficient of rice digestion on STR and DDM. Values are means of triplicate ± the standard error of the mean (SEM).

5.3.8. Panelist preference for cooked rice

Table 7 shows the statistical calculation of Triangle test for difference on two sample of rice with and without addition low acyl gellan gum. The triangle test result shows that among 20 panellists, there were 12 people who had the correct answer to recognise the difference between to type of cooked rice. In accordance with statistical analysis, this data give information that both rice samples were significantly different on the level 5% and no difference on the level 1%.

In accordance to the sensory test, panellist were able to differentiate between two rice samples, meaning there was a change in cooked rice characteristic after the addition of low acyl gellan gum. Furthermore, this result reflected to the next differentiation sensory test. As shown in the Table 8, additional gellan gum changed rice structure in hardness and stickiness.

On average, rice with additional 1% low acyl gellan gum has a higher score in hardness, changing from 'just right' to 'hard'. Low acyl gellan gum was decreasing the stickiness of cooked rice from 'just right' to 'dry'. The slight change in texture will be confirmed for panellists acceptance using organoleptic preferences test.

Table 7. Triangle test result between cooked rice control and rice with additional 1% low acyl gellan gum

n	X	z-value	Z-table	Significance level	
				5%	1%
20	12	2.29	1.64	Yes	No

Table 8. Sensory analysis of cooked rice with score number represent following parameter, for hardness: 1 = very soft, 3 = just right, 5 = very hard; and for stickiness: 1 = very dry, 3 = just right, 5 = very sticky.

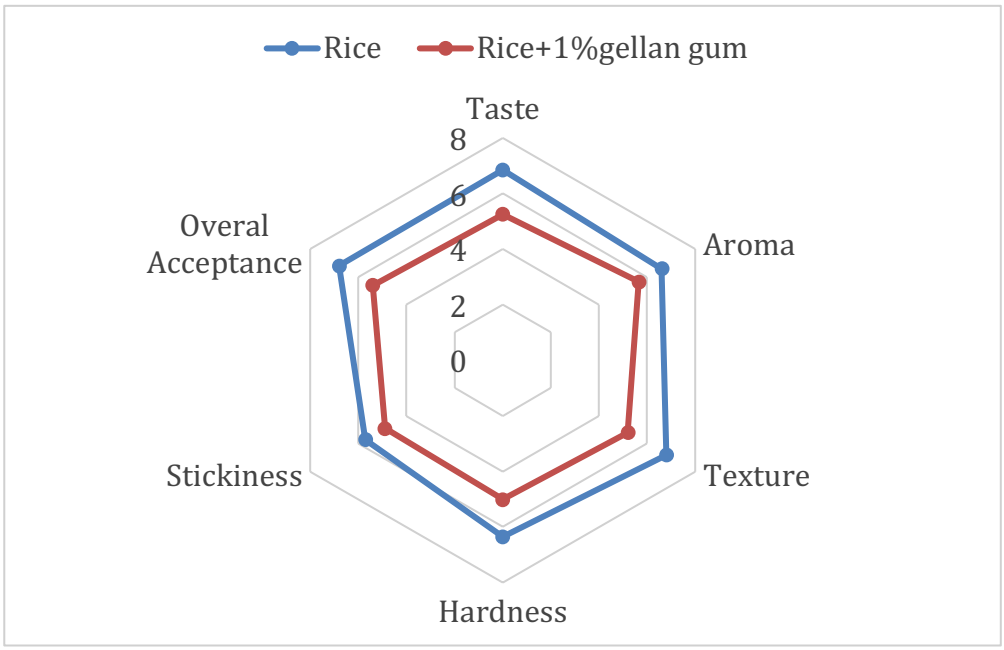
Sensory Analysis	Rice	Rice+1% gellan gum
Hardness	2.80 ± 0.41 *a*	3.85 ± 0.49 *b*
Stickiness	3.25 ± 0.72 *c*	2.05 ± 0.69 *d*

Number presentation are in mean ± standar deviation, different letters in the same row indicate significant differences ($P \leq 0.05$)

Figure 40 shows the panellist preferences on the cooked rice with and without addition low acyl gellan gum. Overall acceptance of panellists were decrease by the additional 1% low acyl gellan gum to cooked rice from 'like' to near 'neutral'. The parameters that given no significant change are stickiness and aroma. On the otherhand, panellists felt that hardness, texture and taste of both sample were different.

Low acyl gellan gum forms firm and non elastic gel after cooling (Sworn, 2009). The 1% solution was able to covering cooked rice and change its structure. The spider-web diagram shows that rice with low acyl gellan gum has lower acceptance on all parameter compared to rice control. However, all panellist gave score ranging from neutral to very like which is rice with low acyl gellan gum was still pleasant to consume.

a)



b)

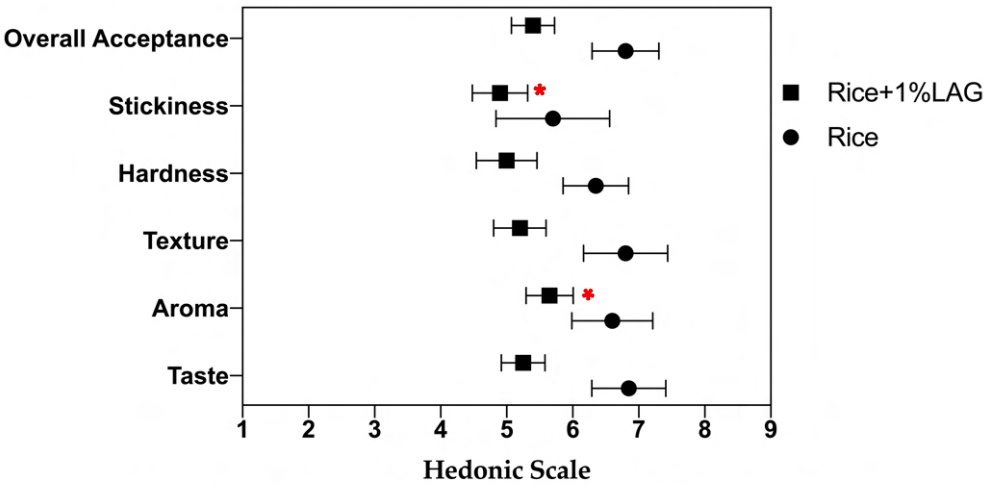


Figure 40. Sensory test for panellist preferences on cooked rice sample using hedonic score, 1 = dislike extremely, 5 = neither like of dislike, 9 = like exetremely. Data presented in spider web graph (a) and plot graph (b). Statistical significance determined using the Holm-Sidak method, with alpha = 0.05. Each row was analyzed individually, without assuming a consistent SD. Number of t tests: 6. Rows marked with red star show no significant different.

5.3.9. Preliminary study of imaging rice digestion using MRI

Although there were initially three healthy volunteers scheduled to participate in the in-vivo MRI study, unfortunately restrictions related to the coronavirus pandemic meant that access to only one healthy volunteer was granted. With the volunteer's consent, the first MRI scan was performed, where consumption of rice without any addition of low acyl gellan gum was monitored. Again, stricter restrictions related to the coronavirus pandemic resulted in this work being left unfinished and open for future experiments, as it became impossible to monitor the consumption of rice with added low acyl gellan gum within reasonable time for this thesis. In this subsection, MRI data from the rice experiment are presented.

The experimental procedures and the rice meals were accepted well by a healthy volunteer who completed the study. On moderately T2 weighted MRI images as shown in Figure 41, in the stomach the water drink appears bright and the cooked rice appears grey. The rice meal appeared to sediment towards the bottom of the stomach with a layer of water at the top. A bolus of rice, likely formed during the swallowing process, can be seen with also fragments of boluses sedimenting in the stomach by gravity.

The preliminary in vivo MRI pilot study of rice meals cooked with and without gellan gum was positive. It allowed to confirm acceptability to human volunteers, to refine the experimental procedures and provided some of the first images of these rice meals in the human stomach. Such data will help us to establish data driven in-vitro/in-vivo relevance of our in-vitro digestion protocols.

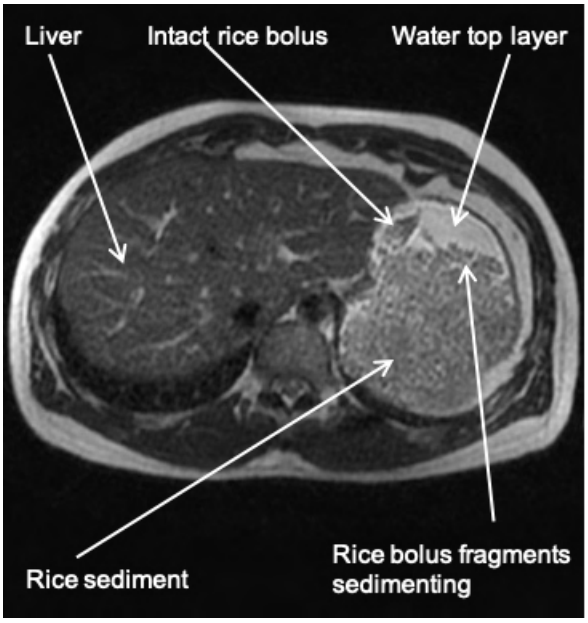


Figure 41. MRI image of rice bolus in the stomach.

5.4. Conclusions

Study on food digestion using *in-vitro* models gains more attention due to the flexibility, safe, convenient and not potentially harmful compare to *in-vivo* model. The *in-vitro* digestion model should be able to mimic the process that occur in the human gut. Our STR models offered simple approach to measure mass transfer from permeable walled cylinder tank to liquid solution outside the membrane. Meanwhile DDM allows mechanical compression that can be set to run contraction and peristaltic movement, similar to appear in human gut system. The comparison of both model was applied to study the effect of additional low acyl gellan gum on rice digestibility.

Our experiment showed that STR gave more destructive effect to the rice structure and DDM static have a less effect. Using all methods of in-vitro digestion in this study show that samples of rice with additional 1% gellan gum have slower digestion rate compared to rice control. In average STR gave the highest digestibility rate and DDM without contraction was the lowest. The use of 2 cpm mode was enhanced the rice digestibility for 29-30%.

On the mass transfer study, contraction 2 cpm has been found giving smaller mass transfer coefficient than DDM without contraction. This phenomena may be occurred due to the compression retarded the flow of recipient liquid, resulting slower diffution rate. However, the sample homogenity during the sampling methods must be taken to the account on DDM experiment.

CHAPTER 6

Conclusions and Future Work

The focus of this study has cored on the in-vitro models to evaluate the digestion rate of rice and rice with additional gellan gum. This investigation was categorized into three main experimental subject including understanding the application three type of in-vitro digestion models (chapter 3), the multiscale study on rice digestion using static model (chapter 4) and the application of unique stirred tank reactor (STR) and dynamic duodenum model (DDM) on kinetic and mass transfer of rice digestion. The main funding of each section are presented in the next sub-section. Final conclusion and suggestion on future work are available in the last section.

6.1. Developing methods

According to this initial experiment, it is possible to use Stirred tanks to characterise some of the phenomena in the nutrient absorption. The DDM acts to mimic nutrient absorption from the small intestine to blood stream. The presitaltic and segmentation movement can be easily to managed based on the research purpose. Clossing the both side of duodenal tube was able to simulate chyme mixing and permeable membrane simulates mass tranfer from inside permeable to outer side.

The Infogest static digestion methods which have gained a concensus as standardised method to use on food matrices samples. This method offers flexible application from powder to different type of foods. The use of simulated fluid in each digestion phase makes the condition similar to the human gut environment. Altough

the mixing and particle size reduction are not representative of the real process in the digestive system.

In this study static and dynamic *in-vitro* models as mentioned before were used on samples ranging from the simplest drug model, glucose solution, semi-solid food (bread and rice) and solid grain matrices. All the methods were used on the experimental study on structured food digestion, especially cooked rice with and without addition of gellan gum.

6.2. Effect of gellan gum on cooked rice digestion

In this works we aimed to study the effect of additional gellan gum on cooked rice structure and its in-vitro digestibility. We used the Infogest method with some modification to process rice samples in-vitro. A multi scale method was used using high resolution DSLR camera and microscopic technique. We developed the methods to evaluate the mechanism of enzyme and intestinal fluid penetration to rice matrix.

Our research has shown that adding 1% low acyl gellan gum to rice cooking could reduce the GI of white rice in in-vitro experiments by 16%. Cooked rice with 1 per cent of low acyl gellan gum has no significant hardness difference than intact jasmine rice based on the texture analysis study. The multiscale study could explain the effect of low acyl gellan gum to protect rice surface from penetration of intestinal enzyme and fluid.

6.3. Application of STR and DDM models in rice digestion

This work aimed to study the effect of additional low acyl gellan gum on rice digestion using an in-vitro digestion model, which could mimic peristaltic and contraction in one duodenum compared to a simple stirred tank model. Based on images analysis, rice particles with the addition of gellan gum appeared to be less broken down compared to the rice control. The least are samples processed using DDM static. In addition, the ESEM technique could examine the rice surface structure and the presence of gel.

All rice samples were digested quickly during the first 30 minutes, and the process in each in-vitro model showed a similar rate. At this point, the percentage of digested rice starch with additional low acyl gellan gum was lower compared to rice control. The digestion process started to hit the plateau after 90 minutes based on the kinetic models and experiment results.

6.4. Suggestion for future work

This study provide insight in several aspects of the effect of addition of low acyl gellan gum in rice digestion that would be interesting to explore. After finding that low acyl gellan gum could inhibit rice digestion in the *in-vitro* model, the further *in-vivo* study will be a great step to investigate. It will be interesting to compare glycaemic index of rice with low acyl gellan gum from *in-vivo* study with this works. Furthermore, as the study of rice digestion on MRI analysis was stopped in the preliminary works, it may be a good finding if further study show how rice with gellan gum behave in the human stomach.

STR and DDM have been shown as potential tools to examine food digestion. However, further study needs to be done to find a better sampling methods. The homogeneous sample may lead to a comprehensive understanding on the food digestion in human small intestine.

Further investigation on incorporates others hydrocolloids in rice cooking will give more comprehensive data to study the effect of hydrocolloids on cooked rice digestibility. For instance, in such investigations, agar powder from seaweed and konjac powder can be employed. These hydrocolloids are frequently used in households to make jelly. The findings will serve as a good reference for preparing low GI cooked rice at home.

Additionally, a potential clinical study involving the measurement of consumers' blood sugar levels could add to the expansion of rice and hydrocolloid digesting knowledge. At least ten participants can be recruited to consume rice with and without hydrocolloids and gradually monitor their blood sugar levels using a glucose test kit (Brouns *et al.*, 2005).

This study mainly evaluate cooked rice with additional low acyl gellan gum. The use of another staple food such as bread will be interesting to study. Bread with additional hydrocolloids might be have a slower digestion rate and improved its functionality as healtier food.

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