PREBIOTIC EFFECTS OF *CUCURBITACEAE* FRUITS USING *IN-VITRO FERMENTATION* BY GUT MICROBIOTA AND DEVELOPMENT OF GRANOLA BARS AND THEIR EFFECTS IN HUMANS

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by

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LIST OF SYMBOLS

a*	Reddish and greenish color
b*	Yellowish and bluish color
c*	Colorfulness
h*	Hue
l	Liter
L*	Luminosity
M	Molar
mL	Milliliter
N	Normal
P	Pressure
V	Suction volume
µL	Microliter
µM	Micrometer

LIST OF ABBREVIATIONS

AACC	American Association for Clinical Chemistry
AOAC	Association of Official Analytical Chemists
AXOs	Arabinoxylan-oligosaccharides
BAC	Bacteroides
BIF	Bifidobacterial
CAM	Complementary and Alternative Medicine
CD	Chrohn's disease
CFU	Colony Forming Unit
CIE	Commission Internatiole de' Eclairage
CLOS	Clostridium
Cu	Kjeldahl catalyst
DF	Dietary fiber
DGGE	Denaturing gradient gel electrophoresis
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DP	Degree of polymerization
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
EU	European Union
EUB	Eubacterium
FC	Flow cytometry
FD	Freeze drying
FISH	Fluorescent *in situ* Hybridization
FOS	Fructooligosaccharides

G	gram
GC	Gas chromatography
GI	Gastrointestinal tract
GOS	Galactooligosaccharides
H	Hour
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
HCI	Hydrochloric acid
HD	Hot air drying
HPLC	High Performance Liquid Chromatography
IBD	Inflammatory bowel disease
IBS	Inflammatory bowel syndrome
IDF	Insoluble dietary fiber
IMO	Isomaltooligosaccharides
IPS	Institut pengajian siswazah
KcI	Potassium chloride
LAB	Lactic Acid Bacteria
Min	Minute
MOH	Ministry of Health
MOS	Mannooligosaccharides
mRNA	Messenger ribonucleic acid
MRS	de Man, Rogosa and Sharpe
N_2	Nitrogen
Na_2HPO_4	di-sodium hydrogen phosphate
$NaH_2PO_4.H_2O$	Sodium phosphate monobasic monohydrate
NaOH	Sodium chloride
NFF	Nutraceuticals and Functional Food

NGS	Next Generation Sequencing
NHMS	National Health and Morbidity Survey
PBS	Phosphate Buffer Solution
PI	Prebiotic Index
PSU	Prince of Songkhla University
qPCR	Quantitative Polymerase Chain Reaction
RFOs	Raffinose oligosaccharides
RGB	Red, green, blue
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SCFA	Short Chain Fatty Acids
SD	Spray drying
SD	Standard deviation
SDF	Soluble dietary fiber
SDS	Sodium dodecyl sulphate
SEC	Scientific Equipment Center
SEM	Scanning Electron Microscope
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SPSS	Statistical Package for Social Science
SSF	Simulated Salivary Fluid
TKN	Total Kjeldahl Nitrogen
TPA	Texture profile analysis
TRFLP	Terminal restriction fragment length polymorphism
UC	Ulcerative colitis
UPMS	Unit Pengurusan Makmal Sains

US	United States
USFDA	United States Food Drug Administration
USM	University Sains Malaysia
VD	Vacuum drying
XOS	Xylooligosaccharides
Cm	Centimeter

LIST OF APPENDICES

KESAN PREBIOTIK TANAM-TANAMAN KELUARGA *CUCURBITACEAE* MENGGUNAKAN FERMENTASI '*IN VITRO*' USUS MIKROBIOTA DAN PERKEMBANGAN BAR GRANOLA DAN KESAN PADA MANUSIA

ABSTRAK

Penyakit radang usus (IBD) adalah kelaziman di negara-negara Barat dan telah kekal selama bertahun-tahun, dan penyakit ini telah meningkat dengan pesat di negara-negara Asia termasuk Malaysia. Kajian ini adalah untuk menyiasat aktiviti prebiotik tumbuhan *Cucurbitaceae* menggunakan penapaian *in vitro* oleh mikrobiota usus. Profil pemakanan dan pencirian fizikokimia setiap serbuk labu kering beku (FDPP), serbuk tembikai musim sejuk (FDWMP) dan serbuk tembikai batu (FDRMP), dan produk formulasinya; bar granola tembikai musim sejuk (GBWM) iaitu kawalan GBWM, GBWM + 4% inulin, GBWM + 8% inulin dan GB inulin 4%, masing-masing, telah disiasat. Ciri-ciri prebiotik semua sampel kering beku dinilai ke atas pertumbuhan yang mempromosikan probiotik *Lactobacillus plantarum* TISTR 1465 dan *Bifidobacterium lactis* BB12. Aktiviti prebiotik sampel ini melalui penapaian in vitro menggunakan kultur kelompok, *Pendarfluor in situ Hybridization* (FISH), skor indeks prebiotik (PI), dan pengeluaran asid lemak rantai pendek (SCFA) sebagai produk akhir penapaian telah dinilai. Kesan prebiotik 4 formulasi GBWM yang diperkuat dengan kepekatan inulin yang berbeza disiasat di kalangan subjek sihat yang menggunakan bar granola 25 g setiap hari dan sampel faecal mereka dikumpulkan pada titik masa yang berbeza. Kepelbagaian mikrob dan tingkah laku buang air besar melalui kajian in vivo telah diperiksa. Pertumbuhan *L. plantarum* TISTR 1465 diperhatikan dalam FDWMP manakala FDRMP dalam *B. lactis* BB12. Pertumbuhan *Bifidobacterial* telah meningkat dengan ketara ($P > 0.05$) dari 0 jam dan

72 jam untuk glukosa D dan FDWMP, bagaimanapun, peningkatan penduduk tidak ketara untuk inulin, FDPP dan FDRMP. Bilangan *Lactobacili* meningkat dengan ketara pada 6 jam untuk FDPP dan 24 jam untuk FDWMP dan FDRMP. Indeks prebiotik glukosa D, inulin, FDPP, FDWMP dan FDRMP ialah 1.33, 1.50, 1.75, 1.90 dan 1.44. Kedua-dua asid asetik dan propionik adalah SCFA utama yang dihasilkan. Kandungan lembapan terendah (3.60%) dikesan dalam GBWM + 8% inulin, tertinggi dalam kandungan abu (1.96%) dipantau dalam kawalan GBWM. Protein tertinggi (8.51%) dicatatkan dalam GBWM + 4% inulin, manakala yang tertinggi dalam jumlah serat pemakanan (27.56 g) ditemui dalam GBWM + 8% inulin. Penerimaan keseluruhan penilaian deria menunjukkan bahawa kawalan GBWM lebih disukai berbanding formulasi lain. Nilai kekerasan kawalan GBWM adalah yang paling rendah atau lebih lembut berbanding formulasi lain yang diperkaya dengan inulin. Untuk profil genus, kedua-dua *Bifidobacterial* dan *Lactobacillus* ditemui dalam semua formulasi kecuali kawalan GBWM kerana kehadiran inulin. Kekerapan pembuangan air besar (1 kali/hari) dan (lebih daripada 1 kali/hari) meningkat selepas penggunaan kawalan GBWM dan GBWM + 4% inulin. Kekerapan masa buang air besar meningkat selepas penggunaan dari semua formulasi kecuali GBWM + 4% inulin. Jenis najis (Jenis 4 normal) di kalangan subjek meningkat selepas penggunaan semua formulasi. Warna najis (Brown) ditingkatkan selepas penggunaan daripada semua formulasi kecuali untuk kawalan GBWM. Jumlah najis setiap masa buang air besar (1-2 najis) selepas penggunaan kawalan GBWM dinaikkan daripada 50% kepada 100%. Secara keseluruhan, penggunaan FDWMP dalam bar granola menyebabkan kenaikan abu, protein, dan jumlah serat pemakanan, peningkatan kekerapan tingkah laku buang air besar, dan diterima dengan baik oleh ahli panel..

PREBIOTIC EFFECTS OF *CUCURBITACEAE* FRUITS USING *IN-VITRO* FERMENTATION BY GUT MICROBIOTA AND DEVELOPMENT OF GRANOLA BARS AND THEIR EFFECTS IN HUMANS

ABSTRACT

Inflammatory Bowel Disease (IBD) is prevalence in Western countries and has remained over years, and the disease has been rapidly increasing in Asian countries including Malaysia. The present study was to investigate the prebiotic activities of *Cucurbitaceae* plants using *in vitro* fermentation by gut microbiota. The nutritional profile and physicochemical characterization of each freeze-dried pumpkin powder (FDPP), winter melon powder (FDWMP) and rock melon powder (FDRMP), and their formulation products; granola bars of winter melon (GBWM) namely GBWM control, GBWM + 4% inulin, GBWM + 8% inulin and GB of inulin 4%, respectively, were investigated. The prebiotic properties of all freeze-dried samples were evaluated on the growth promoting of probiotics *Lactobacillus plantarum* TISTR 1465 and *Bifidobacterium lactis* BB12. The prebiotic activity of these samples through *in vitro* fermentation using batch culture, Fluorescent *in situ* Hybridization (FISH), prebiotic index (PI) score, and production of short chain fatty acids (SCFAs) as fermentation end products were evaluated. The prebiotic effects of 4 formulations of GBWM fortified with different concentrations of inulin were investigated among healthy subjects who consumed a daily 25 g granola bars and their faecal samples were collected at different time points. The microbial diversity and defecation behaviour via *in vivo* studies were examined. The growth of *L. plantarum* TISTR 1465 was observed in the FDWMP while FDRMP in *B. lactis* BB12. The growth of *Bifidobacterial* has significantly increased ($P > 0.05$) from 0 hour and 72 hours for D glucose and FDWMP, however, the increase of population was not significant for inulin, FDPP and FDRMP. The number of *Lactobacili* significantly increased at 6 hours for FDPP and 24 hours for FDWMP and FDRMP. The prebiotic index of D glucose, inulin, FDPP, FDWMP and FDRMP were 1.33, 1.50, 1.75, 1.90 and 1.44. Both acetic and propionic acid were the main SCFAs produced. The lowest moisture content (3.60%) was detected in GBWM + 8% inulin, highest in ash content (1.96%) was monitored in GBWM control. The highest protein (8.51%) was recorded in GBWM + 4% inulin, whereas the highest in total dietary fibre (27.56 g) was discovered in GBWM + 8% inulin. The overall acceptance of sensory evaluation showed that the GBWM control was more preferred over other formulations. The value of hardness of GBWM control is the lowest or softer compared to other formulations fortified with inulin. For genus profile, both *Bifidobacterial* and *Lactobacillus* were discovered in all formulations except for GBWM control due to presence of inulin. The frequency of defecation (1 time/day) and (more than 1 time/day) were increased after consumption of GBWM control and GBWM + 4% inulin, respectively. The frequency of defecation time was increased after consumption from all formulations except for GBWM + 4% inulin. The type of faeces (Type 4 normal) among subjects was increased after consumption of all formulations. The colour of faeces (Brown) was increased after consumption from all formulations except for GBWM control. The amount of stool per defecation time (1-2 stools) after consumption of GBWM control was increased from 50% to 100%. In overall, the usage of FDWMP in granola bars resulted in increment of ash, protein, and total dietary fibre, improvement of regularity of defecation behaviour, and well accepted by panellists.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The gut microbiota has approximately 100 trillion microorganisms which bacteria is the major component with high population density, followed by a wide diversity of hundreds of yeast species and thousands of subspecies of parasites (Gomes *et al.*, 2014; Jia *et al.*, 2008). The gut microbiota is essential in normal digestive functions of the host, maturation of human immunity, brain development and defence against pathogens (Heijtz *et al.*, 2011; Maslowski and MacKay, 2011). Furthermore, gut microbiota can maintain the bile acid profile that can affect the digestion and metabolism of the host (Sayin *et al.*, 2013). Host diseases such as obesity, colorectal cancer and inflammatory bowel disease (IBD) are caused by imbalanced of gut microbiota (Gilbert *et al.*, 2018; Postler and Ghosh, 2017).

Functional food refers to any traditional food or food products after modification or fortification that can offer improvement in the human body due to nutrient value added (Santini *et al.*, 2017; Shahidi, 2012). According to Roberfoid (2000), functional food refers to those food products that have the potential to give wellness and good health, physiological functions but not cure the disease. The researcher added, one of the purposes of consuming functional food is to prevent some chronic diseases such as obesity, cancer, diabetes and cardiovascular disease. In another statement by Balthazar *et al.*, (2017), the two main ingredients that the food industry are using such as probiotics and prebiotics, which are widely known to offer many health benefits including to modulate human host health, thus the gut microbiota composition can be improved. This statement has been supported by Iwatani and

Yamamoto, (2019), in their review, consuming probiotic *Lactobacillus*, oligosaccharide and dietary fibre can improve gut health. The combination of prebiotics and probiotics are known as symbiotics, help to benefit the host by extending the survival rate of microbial supplements into the gastrointestinal tract (Ouwehand *et al*., 2007).

The two groups of dietary fibre (DF) are soluble dietary fibre (SDF) and insoluble dietary fibre (IDF). SDF has caught attention from researchers due to its prebiotic effects and technology applications (Mrabet et al., 2017). The components of SDF are mainly non-cellulosic polysaccharides which can be classified as pectin and mucilage, while IDF is composed of cell wall components that consist of cellulose and hemicellulose (Dai and Chau, 2017). Among Malaysians, a recommendation made by Malaysia Dietary Guidelines (MDG) regarding the intake of dietary fiber shall be between 20 to 30 g, for both men and women according to the Recommended Nutrient Intakes for Malaysia (National Coordinating Committee on Food and Nutrition, 2005).

Several health benefits associated with consuming high intakes of DF are decreasing the risk of cardiovascular disease and type 2 diabetes, improving gut flora health, controlling appetite and body weight (Adam *et al*., 2016). Apart from that, further high intakes of DF can produce short chain fatty acids (SCFA), thus, improving the immune functions and preventing colon cancer (Dai and Chau, 2017). The prevention of colon cancer can be explained by IDF's ability to bind with several harmful chemicals, such as mutagens and carcinogens, allowing these harmful substances to be eliminated through faeces, whereas SDF has the ability to produce SCFAs through fermentation in the lower colon using probiotic bacteria (Prasad and Bondy, 2019). Meanwhile, higher intake of SDF can enhance satiety, stimulate the release of appetite suppressing hormone, and combine cholesterol with faeces during digestion, which has a positive impact on the health of host metabolism after fermentation in the intestine (Qian Li *et al*., 2017).

Prominent researchers have indicated that increasing the intake of dietary fibre is effective in reducing cardiovascular disease, type 2 diabetes and colorectal cancer (McRae, 2017, 2018a, 2018b). Other common reasons for a health care professional to prescribe dietary fibre supplementation are for patients with constipation (Tresca, Amber, 2021) or gastrointestinal disorders, such as IBD (Basnayake, 2018; Hadley and Gaarder, 2005), or to improve weight loss in diabetic patients who are on treatment (Alfieri et al., 1995).

Cucurbits plants are high in dietary fiber, Beta-carotene (provitamin A), potassium, and vitamin C. Apart from that, cucurbit plants are fleshy, edible, and pericarp with a sweet taste, such as rock melon, water melon, and honeydew, whereas starchy ones are gourds, pumpkins, and squashes (Gebhardt *et al*., 1982).

In recent years, agriculture, food processing, pharmaceutical and feed industries have all taken a growing interest in pumpkin fruit and pumpkin-derived products due to the nutritional and health-protective value of the seed proteins and oil, as well as the fruit polysaccharides (Sojak and Głowacki, 2010). Pumpkin is a healthy source of carotene, pectin, mineral salts, vitamins, and other health-beneficial substances (Jun *et al*., 2006). Previous study by Caili *et al*., (2007), they reported pumpkin (Cucurbita maxima), a high-fiber plant, is recognized to have anti-diabetic properties.

Winter melon or scientifically *Benincasa hispida* or a Malay name (*Kundur*), has been examined as anti-obesity by (Kumar and Vimalavathini, 2004). For the first time, researchers have revealed the potential of winter melon as an anorectic behaviour, most likely mediated via the central nervous system without affecting gastric emptying. An anorectic behaviour refers to a condition where an appetite is decreased. The outcome of the study showed that the mice reduction in food consumption has no link to gastric emptying, where gastric emptying of food has a relationship to overeating and obesity (Cifuentes *et al*., 2021). As the result indicated, winter melon has the property of an anti-obesity agent due to decreased food intake (Kumar and Vimalavathini, 2004).

There are seven sub-species of *Cucumis melo*. or rock melon known as *Cantaloupensis*, *Reticulatous*, *Inodorous*, *Flexuosus*, *Conomon*, *Chito* and *Dudaim* (Boyhan *et al*., 2014). The amount of beta-carotene or pro-vitamin A can be found highest in rock melon, an essential nutrient required for eye health, and may have the ability as an antioxidant to reduce the risks associated with cancer, heart disease, and other diseases (Athirah *et al*., 2018).

Drying is a well-known method for prolonging the shelf life of food products by lowering water activity (a_w). Low a_w values (less than 0.3) are good for powder stability and have a longer shelf life because there is less free water available for biochemical activities (Sahni et al., 2014). Minimizing a_w is important for the continued viability of living organisms throughout the production, storage, transit, and consumption processes (Wang et al., 2020; Vesterlund et al., 2012). The freeze-drying procedure stabilizes the product's structure and nutritional content while preserving all of its health benefits for the body, the digestive system, and the intestines. Freeze drying is the best method for dehydrating natural products that contain components that are thermally sensitive and prone to oxidation because it takes place in a high vacuum and at low temperatures (Sablani et al., 2019; Duan et al., 2016; Bhatta et al., 2020).

According to earlier research, freeze-drying has already been shown to be an effective method for dehydrating a wide variety of fruits and vegetables, namely asparagus, apple, blackberries, coffee, garlic, ginger, guava, strawberries, pumpkin, tomatoes, tea, ginger, and maple syrup (Bhatta et al., 2020). Freeze drying is the most practical and efficient method for producing dried synbiotics and preparing prebiotics like inulin and gum acacia (Dhewa et al., 2011). However, no study has examined and compare the prebiotic property of freeze-dried pumpkin, winter melon and rockmelon, respectively.

1.2 Problem statement

IBD is a chronic disease that is identified from the condition of relapsing and remitting inflammation in the gastrointestinal (GI) tract. IBD is common in Western countries and has remained relatively stable over years, however, the disease has been rapidly increasing in Asian countries. There are two major factors that contribute to IBD; urbanization and a shift toward a Western diet. Colorectal cancer is a long-term complication of IBD (Yusof *et al*., 2013). Researchers added, in Malaysia, several factors are linked to cause colorectal cancer, namely, consistent eating outside the home, high use of seasoning and preservatives in food. Besides that, low intakes of fibre in a meal diet can cause the prevalence of colorectal cancer, hence, it is strongly recommended to include a diet that is high in DF (Chuang *et al*., 2012).

In a study by Ong *et al*., (2018), the use of complementary and alternative medicines (CAM) practices with IBD in Singapore and Malaysia are probiotics, vitamins/minerals (vitamin D, multivitamins, vitamin C, iron), food-based therapies namely fish oil, aloe vera, barley, turmeric, green tea; and traditional Chinese medicine/traditional Indian medicine (ayurvedic) and traditional Malay medicine (*jamu*) or mind/body practices (*Chinese qi gong*).

Besides the mentioned disease above, diabetes mellitus (DM) is a category of chronic metabolic disorders or diseases in which the amount of blood glucose (hyperglycaemia) rises persistently (Mirmiran *et al*., 2014). In Malaysia, from 2015 to 2019, the statistics of Malaysian adults having diabetes, obesity and hypertension have increased. The outcome from the National Health and Morbidity Survey (2019), revealed that the percentage has increased from 13.4% in 2015 to 18.3%, with regards to diabetes described as 7.0 mmol/L or above. The data of gaining weight among Malaysians has increased as well, from 48.6% in 2015 to 52.6% in 2019; hence, Malaysians are gradually increasing in their weight. Obesity rates among Malaysian adults have also increased, rising from 17.7% (or 3.3 million people) in 2014 to 19.7% in 2019. As of 2019, half of Malaysian adults (50.1%) were overweight (Codeblue, 2019). Past researchers have shown that functional foods play a crucial role in the management of diabetes mellitus by enhancing human and animal health conditions (Alkhatib *et al*., 2017; Bahadoran *et al*., 2013; Mirmiran *et al*., 2014).

Defecation, bowel movement or bowel habit refers to the discharge or passage of faeces from the large intestine. The amount of defecation time an individual discharge daily is known as the frequency of bowel movement. Usually, bowel movement between individuals is three times per day to three times per week. However, defecation three times per week is considered suffering from constipation (Ambartsumyan and Rodriguez, 2014) while over three times per day is diarrheal (Rao *et al*., 2014).

1.3 Rationale and significance of the study

The way in which the intake of these foods influences the gut microbiota and how the microbiota's metabolic activity affects the host's health and well-being is increasing interest in studying the prebiotic effect of foods. Food matrices are made up of a variety of nutrient and non-nutrient ingredients that interact complexly. With this in mind, foods have a basic feeding function, with some foods, such as functional foods, providing health benefits that go beyond nutrition. In addition to essential nutritional functions, functional foods can have metabolic benefits and/or minimise the risk of chronic diseases. They can have a similar appearance to traditional foods and may be consumed as part of a daily diet. Functional foods include prebiotics.

Many by-products have recently been investigated as low cost and alternative prebiotic sources. Thuaytong and Anprung (2011) used probiotics *Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* BB-12 in Man Rogosa Sharpe (MRS) broth containing 1% glucose, 1% inulin and 1% red and white guava fruit. The findings revealed that both substrates were capable of supporting the growth of bacteria.

Since there is a high demand for functional foods that can assist in health maintenance, including prebiotics in industrialized foods has become a viable and healthy choice. Furthermore, adding prebiotics to food products can provide numerous benefits to the food industry, including improved sensory characteristics, improved nutritional composition and longer shelf life. Prebiotics are commonly used in baked goods, breakfast cereals, drinks (such as fruit juices, coffee, cocoa and tea), dairy products (yoghurt, cheese and milk), table spreads, buttery products and desserts (ice cream, puddings, jellies and chocolates). Prebiotics also have gelling properties, namely inulin, which maintains the stability of emulsion, provides spreadable texture and retains water (inulin and fructooligosaccharides-FOS) to enable the development of low-fat with a good taste and texture. In addition to that, consumers enjoy convenient and safe options like low calories, low fat and high fibre. Thus, one solution to increasing dietary fibre intake is to consume foods that are high in dietary fibre content, such as granola bars and cereals. Meanwhile, inulin's use in foods has increased in the last ten years, due to its prebiotic content and technological properties. The range of foods fortified with inulin is extensive, with concentrations ranging from 0.75 to 50% depending on the type of food. Inulin's prebiotic effect is undeniably established.

Winter melon is highly valuable for its use as food as well as having a high nutritional value and remarkable medicinal properties. Winter melon has been used in Taiwan as an ingredient for sweets, jams and beverages. Winter melon is either eaten in curry, sugar-coated or syrup-coated or processed as candy in Malaysia, India and Cuba. Winter melon can be explored for use in a variety of food items, such as jams, juices, drinks, cakes and ice creams, which may target certain groups of people, for example, regular and healthy people, diabetic patients, and others. Older generations in Asia typically either eat winter melon in raw form or cook it without much knowledge of the benefits of the fruit.

While preliminary studies have been conducted in laboratory experiments by past researchers with anti-ulcer, anti-obesity, anti-diarrheal, anti-inflammatory, anti-nociceptive and anti-pyretic effects of winter melon fruit, there is a lack of information, especially about the prebiotic properties in this fruit. As a result, the prebiotic activities of cucurbit fruits (pumpkin, winter melon and rock melon) are investigated in this study using *in vitro* fermentations and *in vivo* human trials (consuming food product) that are beneficial to gut microbiota. Hence, among all the nutritional properties of inulin, its health benefits as a dietary fibre fortified into granola bars of winter melon were analysed to be considered as a functional food product.

1.4 Objectives of the study

1.4.1 General objectives

The objectives of the study are divided into 3 phases. The first phase is to determine the physicochemical profiles and prebiotic properties of freeze-dried powder of pumpkin, winter melon and rock melon, respectively in promoting probiotics growth.

The second phase is to analyse their prebiotics activities using *in vitro* fermentation batch culture while the third phase is to develop a food product (granola bars fortified with different concentrations of inulin) and tested on *in vivo* human trials and analyse the microbial diversity as well as the defecation behaviour.

1.4.2 Specific objectives

1. To determine the proximate composition of each freeze-dried powder of pumpkin (FDPP), winter melon (FDWMP) and rock melon (FDRMP), respectively from the cucurbit family.

2. To evaluate the potential prebiotic properties of FDPP, FDWMP and FDRMP and also carbon sources on the growth promoting of probiotics (*L. plantarum* TISTR 1465, *Bifidobacterium subsp.* animals *lactis* BB12) and simulation of gastrointestinal digestions on the simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

3. To investigate the prebiotic properties of pre-digested samples obtained by simulated gastrointestinal digestion digestion via *in vitro* fermentation in batch culture, analyse their gut microbiota composition through identification of population changes of major bacterial groups using Fluorescent *in situ* Hybridization (FISH) technique and determine their growth of beneficial and pathogenic bacteria relationship using Prebiotic Index (PI) equation, as well as the productions of Short Chains Fatty Acids (SCFAs) using High Performance Liquid Chromatography (HPLC).

4. To develop a food product; granola bars of winter melon (GBWM) fortified with inulin, and to determine its proximate composition, sensory evaluation, morphological characterization using Scanning Electron Microscope (SEM), colour and the texture profile.

5. To analyse the bacterial gut population of faeces from subjects (Baseline, Ingestion and Evacuation Week) from consumption of GBWM fortified with inulin using Next Generation Sequencing (NGS).

6. To examine the defecation behaviour (bowel movement regularity) of subjects (Baseline, Ingestion and Evacuation Week) from consumption of GBWM fortified with inulin based on the set of questionnaires.

CHAPTER 2

LITERATURE REVIEW

2.1 Cucurbit family

The family of gourds, *Cucurbitaceae*, contains more than 900 plant species known as the gourds and cucurbits. Cucumbers, melons, watermelons, pumpkins, squashes and many more are all included. For culinary purposes, at least 20 species are used, which typically involve consumption of the flesh of mature fruit, whole immature fruit and/or seeds. Three species; *Cucumis* (cucumbers, melons), cucurbits; (pumpkins, squash) and *Citrullus* (watermelons), are among the top ten most economically important vegetables in the world, while the remaining species are important to their respective regions. Cucurbits are grown for ornaments, containers, and medicinal applications in addition to consumption (Schaffer and Paris, 2016).

Cucurbits can be cultivated in almost all parts of the world. Cucurbits are divided into cultivated and wild cucurbits. The majority of cucurbit species are mesophytes with large palm leaves, fibrous root systems and, notably, fruits. Wild cucurbits are typically small, round, and green in colour, with a bitter taste due to alkaloid compounds known as cucurbitacin. A cucurbit fruit contains hundreds of seeds. In cultivated form, they have larger leaves and sizes, thicker roots, fewer branches and seeds. Cultivated plants may not always have fruits in a round shape, but cylindrical. Cultivated cucurbits have various colours and fruit patterns. The flesh of fruits belonging to cultivated is sweet, not bitter, higher in fibre, starch, sugar and carotenoids (Schaffer and Paris, 2016).

There are five sub families of cucurbit; *Fevillaeae*, *Melothrieae*, *Cucurbitaceae*, *Sicyoideae,* and *Cylanthereae*. The main cultivated genera, namely

Cucurbita L., *Cucumis L*., *Citrullus L*., *Lagenaria L*., and *Luffa L*. *Luffa L*. is found in the sub family *Cucurbitaceae* while *Sechium L*., found in the sub-family *Sicyoideae* (Whitaker and Davis, 1962).

Cultivated cucurbits differ in fruit characteristics despite being similar in ground development and root habit. For example, summer squash is eaten during the immature stage while watermelon is eaten when mature. Squash can be baked; cucumbers can be pickled and eaten as salad, watermelon can be candied and freshly eaten, while melon can be eaten as dessert. While squash, pumpkin and chayote, their seeds, flowers and roots are consumed by humans. Other parts of cucurbits are used for storage, bottles, utensils, drinking containers, smoking pipes, musical instruments, gourd craft decoration, masks, and floats for fish nets, in addition to food. While loofah fruit, its mature fibre can be used as a sponge for personal hygiene, filtration and household cleaning (Robinson and Decker-Walters, 1997). In China, *Cucurbita moschata* or butternut squash has been used as a traditional medicine and also as a healthy food. At the time, Chinese societies believed that this fruit was beneficial to the spleen and lungs (Jiang and Du, 2011; Quanhong *et al*., 2005). Cucurbit fruits are depicted in Figure 2.1 below.

Figure 2.1 Cucurbits fruits (Source: https://pixabay.com)

2.1.1 Pumpkin (*Cucurbita moschata*)

The pumpkin is a squash plant family member that is round in shape with seeds and pulp inside and is yellow to orange in colour. The origin of the fruit is from North America and the oldest pumpkin seeds were discovered in Mexico between 7000 and 5500 BC. After Mexico, Argentina and Chile were the next countries that discovered pumpkin before eventually spreading to Europe and Asia. In brief, pumpkins are planted in almost all parts of the world (Yadav *et al*., 2010).

Pumpkin is a common crop planted in farms ranging from one to ten acres in Malaysia, and its two main species are *Cucurbita moschata* and *Cucurbita moschata Duchesne*. Among the locals, these two species are known as "*labu manis*" and "*labu loceng*". *Cucurbita moschata* are planted in almost every state, while *Cucurbita moschata Duchesne* are planted only in Kedah and Sarawak. The colour of young pumpkin is green while older ones are pale yellow. The flesh is 3 centimetres thick and has a sweet taste (Yok *et al*., 2016). Malaysia encouraged pumpkin cultivation to tackle the issue of abundance due to their short growing period between two to three months (Anem, 2017). The wax that covers the skin protects the fruit from spoilage for up to six months. In the Malaysian market, the current price of pumpkin (*Cucurbita moschata*) is around RM1.50 to RM2.00 per kilogram and each fruit may weigh between three to five kilograms (Mumpung, 2014). Pumpkin is one of the export crops in Malaysia and it contributes to the national income and is an important crop in the agricultural sector.

In a statement by Masdek and Muhammad (2016), a few factors associated with increasing demand for pumpkin are mainly because of the growing population, elevated household income and awareness of consuming healthier food choices. The beneficial nutrients in pumpkin are potassium, vitamin C, fibre and a variety of phytochemicals. Meanwhile, pumpkin possesses several substances that can give benefits to health (Centers for Disease Control and Prevention, 2011). This statement has been supported by Yok *et al*., (2016), who reported that pumpkin can function as anti-diabetic, anti-carcinogenic and anti-microbial potentials. Moreover, the formation of kidney stones can be prevented, and the effects of blood clotting can be reduced by consuming pumpkin.

Due to its high level of fiber and carotenoids, including beta carotene, lutein, and violaxanthin, or its high vitamin C content and low caloric value (Biesiada et al., 2009; 2011; De Carvalho et al., 2012), pumpkin can be a nutritious and helpful addition to a variety of cuisines and fruit products (Nawirska *et al*., 2012). The polysaccharides caught more attention among the nutrients in pumpkin. The polysaccharides of the pumpkin are made up of galactose, glucose, arabinose, xylose and glucuronic acid. The macromolecular compounds with significant biological functions are water-insoluble, but organic solvents soluble (Maran *et al*., 2013; Song *et al*., 2012; Yang *et al*., 2007). The pumpkin polysaccharides have the biological effects of detoxification, antioxidation, reducing blood pressure, lowering blood lipids and lowering cholesterol levels (Nara *et al*., 2009; Wang *et al*., 2012).

2.1.2 Winter melon (*Benincasa hispida*)

Winter melon is an important crop cultivated in China and also in selected countries of the Eastern and Southern parts of Asia (Schaffer and Paris, 2016). Other names of winter melon are ash gourd, ash guard, winter gourd, white pumpkin, wax gourd, white gourd, tallow gourd, gourd melon and Chinese watermelon. Winter melon belongs to the family member of *Cucurbitaceae* and is a well-known crop due to its nutritional uses and medicinal elements (Zaini *et al*., 2011). Researchers added, in Malaysia, winter melon is represented by two cultivars of round and oval, and another type is hybrid round (developed through breeding of the green winter melon genotype and fuzzy white gourd genotype) is also grown.

On average, there's approximately 96.3% moisture, 3.5°Brix, 0.12% acidity and 0.5% minerals in the edible portion of mature winter melon (Sahu *et al*., 2015; Shinde *et al*., 2016). The mineral content (mg/kg) in winter melon is as follows; sodium (268.00 ± 0.02), calcium (99.40 ± 0.1), iron (3.20 ± 0.02), zinc (1.30 ± 0.01), potassium (1.10 ± 0.05) and manganese (1.10 ± 0.01). Minerals are essential for efficiency and maintenance of the body's water balance (Hanif *et al*., 2006). Sodium helps to maintain the fluid balance in the body. Calcium and potassium help to maintain stable blood pressure (Bello *et al*., 2014). Iron helps bring oxygen to a brain deficit that causes behavioural disorders and affects memory (Kim and Wessling-Resnick, 2014). The deficiency of zinc is associated with malnutrition and is necessary for intracellular activities with catalytic, structural and regulatory function in the body (Roohani *et al*., 2013).

This fruit has some similar characteristics to *Cucurbita moschata*, such as large size, yellow flowers and leaves. Apart from that, the unripe winter melon is hairy and the mature one is covered with white wax. There are two shapes of winter melon: round and cylindrical. The colour of the winter melon is green with white and crispy flesh on the inside. Both unripe and mature winter melon can be used for cooking purposes, pickled, and sometimes eaten raw (Schaffer and Paris, 2016).

The sugar content of winter melon is generally low (1% to 2%, w/v) which makes it a suitable candidate for low-calorie juices or drinks. It is also high in phenolic amino acid and nucleosides, which contribute to antioxidant activity and memory loss reduction in humans. The dietary fibre content of winter melon (27.5% of the dry weight) is relatively high, which contributes to help in digestion, promote bowel movement, prevent constipation, and enhance gut health (Sun *et al*., 2017).

Winter melon has high levels of dietary fibre and lipids, which induce interaction between dietary fibre-associated polyphenols and sugar, making them more bio accessible (Palamthodi *et al*., 2019). The content of dietary fibres of winter melon has a strong prebiotic activity (Sreenivas and Lele, 2013). Phenolics, sterols and glycosides of the winter melon may be employed to treat epilepsy, ulcers and other nervous disorders as functionally essential biomass and medicinal compounds. The winter melon's antacid activity helps to preserve pH in the body and counteracts the acidity induced by certain foods (Grubben and Denton, 2004).

2.1.3 Rockmelon (*Cucumis melo* L.)

Rockmelon, cantaloupe or muskmelon originated from the *Cucurbitaceae* family and are commercially important crops in some countries. There are two subspecies: *C. melo* ssp. *melo* and *C. melo* ssp. *agrestis*, characterized by long and spreading hairs versus short hair on the ovaries (Schaffer and Paris, 2016). Rockmelon produces an orange yellowish colour as well as sweet substances. Carbohydrates are abundant in rock melon. The cell wall of rock melon's polysaccharides contains pectin, cellulose and hemicellulose (Lester, 1997). Rockmelon has a very rich nutritional compositions namely, high in Vitamin C or ascorbic acid, which is necessary for growth, bone health, and infection control (Pehlivan, 2017), also Vitamin A, carotene, folic acid, potassium, including Beta carotene which is a pigment in the carotenoid group with antioxidant activity (Lester and Hodges, 2008; Saftner *et al*., 2006).

The moisture content of the flesh is high while the seeds are rich in oil and protein, ascorbic acid, folic acid and potassium. The natural sugars are sucrose, glucose and fructose (Hubbard *et al*., 1989). Rock melon has no cholesterol and is low in fat, sodium, vitamin E, folic acid, iron and calcium (Lester, 1997). Rockmelon is an excellent source of bioactive substances for humans (Petkova and Antova, 2015) and the three compounds such as cucurbitacin-β, lithium, and zinc, are functioning in preventing cancers, fighting depression, dandruff, ulcers and stimulating the immune system (Lester, 1997).

As far as melon production is concerned, based on FAOSTAT data from 2008 to 2016, the main producer was Asia with about 74% of world production (31, 166, 896 tons), followed by America with 11.9% and lastly, Europe with 7.2% (FAOSTAT, 2017). In Malaysia, rock melon is a well-known and favourite fruit, especially "*Golden Langkawi*" with its striking golden yellow colour. Rock melon easily get bruises and split; thus, the fruit pickers must know how to handle the fruit. The level of sugar content will stop increasing once rock melon has been picked.

2.2 Methods of drying

Drying is a vital technique to prolong a food product's shelf-life (Neta *et al.*, 2019). Drying is one of the aged and advantageous methods of preserving all kinds of food such as grains, fruits and vegetables. The consistency of the dried product depends heavily on the conditions of the drying process. During drying, various important features of food change due to moisture loss from the inner structure to the external environment (Mercier *et al.*, 2011). Different drying techniques, such as hot-air drying (HD), vacuum drying (VD), freeze drying (FD) and spray drying (SD) have been commonly used in industrial applications for the preservation of dehydrated plant items (Neta *et al.*, 2019; Yan *et al.*, 2019). Previous research has shown that certain strains of probiotics can withstand the freezing drying process better. For instance, freeze-dried yoghurt can help to maintain a sufficient quantity of viable probiotics (Capela *et al.*, 2006). The freeze-drying yoghurt also retains the yoghurt in a stable powder shape on a high-quality shelf (Kumar and Mishra, 2004).

2.2.1 Freeze drying

Amidst of the drying methods which are currently used in the food processing industries, freeze drying is one of the most advanced methods for heat-sensitive drying of high-value products. Freeze drying avoids excessive shrinkage and creates products with high porosity, consistent quality of nutrients, better taste, aroma, preservation of flavour and colour, enhance the properties of rehydration, better than those drying with conventional technique (Prosapio and Lopez-Quiroga, 2020). Freeze drying is accomplished in two steps; first, the product is frozen, and then, by sublimation, the ice is extracted directly from the solid to the vapour process. During freeze drying, sublimation of the ice creates major changes in the food product's structure and volume.

Depending on the conditions of the process, the ice crystals which sublimated form pores or gaps of different characteristics. The lack of air prevents the degradation of the substance due to oxidation or chemical alteration (Tan *et al*., 2021). In a previous study conducted by Yan *et al*., (2019), the bitter gourd (*Momordica charantia L*.) was freeze dried, and previously sliced and frozen at -20°C for 24 hours and then freeze dried at -50°C. The drying process lasted until the moisture content of bitter gourd slices decreased to around 6% (wet basis). The bitter gourd was ground into powder after drying, passed through a 60-mesh sieve, and sealed prior to use in airtight plastic bags.

2.3 The chemical compositions of *Cucurbitaceae* that related with prebiotics/probiotics

As a result of their antioxidant properties, the majority of *Cucurbitaceae* vegetables have a rich chemical composition that enhances the food health and stability (Bisognin, 2002). The *Cucurbitaceae* family as a whole offers many health advantages, but depending on where it is grown, each plant has a unique set of qualities and affects people's health in a particular way. The *Cucurbitaceae* family has made a big economic impact and is used in many food products, such as important sources of dietary fiber, as well as in other industries including cosmetics. Cucumber extracts, which are known for their cooling, healing, and relaxing characteristics, are frequently used in skin care products. Furthermore, mature fruits of *Luffa aegyptiaca* Mill. have been known since ancient times as natural sponges [Ajuru and Nmom, 2017], which give a variety of beneficial characteristics and aid in insulinemic response and changes in intestinal function. Cucurbits' beneficial qualities make them beneficial for lowering cholesterol, improving insulin sensitivity, and preventing constipation [Rolnik and Olas, 2020].

The *Cucurbitaceae* family's chemical composition contains phytochemicals that have no nutritional value and occur naturally in plants. Tannins, sugars, saponins, polyphenols, and cardiac glycosides are some of the compounds discovered in these plants (Rajasree et al., 2016). In addition, *Cucurbitaceae* has high concentrations of other bioactive compounds such triterpenes, sterols, alkaloids, and carotenoids. Cucurbits have antioxidant properties due to the several of bioactive compounds that can scavenge free radicals, such as cucurbitacins B and E, ellagitannins, and carotenoids like Beta carotene, lutein, and zeaxanthin [Rajasree *et al*., 2016; Grover and Yadav, 2004; Aeri *et al*., 2015].

2.3.1 Colonic microflora and fermentation

The industry of functional probiotic foods is currently very interested in the development of prebiotic food ingredients (Gibson et al., 2017). The two main prebiotics which are fructooligosaccharides (FOS) and galactooligosaccharides (GOS), have the ability to boost microbiota and improve health (Walton et al., 2013). In addition to FOS and GOS, prebiotics found naturally in fruits and vegetables have also been studied. Apart from that, the fiber-rich byproducts of food processing have been demonstrated to benefit the gut flora (Yu et al., 2023).

Although every prebiotic is a fiber, not every fiber is a prebiotic. For a food ingredient to be called a ‘prebiotic’, it requires scientific demonstration that must show the following qualities, namely; resists gastric acidity, hydrolysis by mammalian enzymes and absorption in the upper gastrointestinal tract; is fermented by the intestinal microflora and selectively stimulates the growth and/or activity of intestinal bacteria potentially associated with health and well-being (Gibson and Roberfroid, 1995).

Dietary fiber and prebiotics both have essential mechanisms of action that involve gut microbiota alterations and fermentation in the colon. One of the most diversely colonized and metabolically active organs in the human body is the large intestine (Gibson et al., 2010). The colon contains up to 1000 different types of bacteria, with microbial communities containing roughly 10^{11}-10^{12} cfu/g of contents. The colon is a good place for bacteria to grow due to its slow transit time, readily available nutrients, and has a good pH (Cummings and Macfarlane, 1991). Generally, bacteria having an almost exclusive saccharolytic metabolism (i.e., no proteolytic activity) can be considered potentially beneficial. Such a metabolic profile is typical for lactobacilli and bifidobacteria.

Together with the gut immune system, colonic and mucosal microflora make up a big part of the barrier that keeps pathogenic bacteria from getting into the GI tract. The intestinal flora gets energy from carbohydrates that were not digested in the upper gut. Endogenous substrates (such as mucus) and dietary carbohydrates that are not absorbed in the upper GI tract are the primary substrates. These include resistant starch, non-starch polysaccharides (like celluloses, hemicelluloses, pectins, and gums), non-digestible oligosaccharides, and sugar alcohols. The main fermentation pathway generates pyruvate from hexoses in the undigested carbohydrate. Colonic bacteria utilize an array of carbohydrate hydrolyzing enzymes to generate hydrogen, methane, carbon dioxide, SCFAs (primarily acetate, propionate, and butyrate), and lactate. Certain colonic bacteria generate energy from these fermentation products. Dietary components that stimulate fermentation led to an increase in bacterial mass and consequently fecal mass and, thus have a stool bulking effect. It is estimated that for every 100 g of carbohydrate fermented, around 30 g of bacteria are created (Slavin, 2013).

2.3.2 The mechanism of how intestinal microbiota members to convert dietary carbohydrates to healthy short chain fatty acids

Fermentation and SCFA production are crucial to colonic and systemic health. Even when competing substrates such as glucose and glutamine are available, colonic epithelial cells preferentially utilise butyrate as an energy source. Butyrate is regarded as a crucial nutrient in determining the metabolic activity and growth of colonocytes and may serve as a primary protective factor against colonic disorders, despite contradictory data (Lupton, 2004). SCFAs are absorbed into the bloodstream and are water-soluble. Acetate is systemically metabolized by the brain, muscles, and tissues while propionate is excreted by the liver and may reduce hepatic cholesterol production by inhibiting its synthesis. It is believed that transport to and subsequent metabolism of SCFAs in the liver, muscle, or other peripheral tissues contribute approximately 7–8% of the daily energy requirements of the host (Cummings and Macfarlane, 1991; Gordon et al., 2011). Fermentation and SCFA production also prevent pathogenic organism growth by lowering luminal and fecal pH. Low pH slows down the breakdown of peptides and the formation of toxic compounds like ammonia, amines, and phenolic compounds as well as decreasing the activity of undesirable bacterial enzymes.

2.4 Diversity in gut bacterial community

In the human intestine, a complex population of microorganisms is found such as fungi, parasites, viruses, archaea and primarily bacteria, called gut microbiota (Sommer and Bäckhed, 2013; Thursby and Juge, 2017). Based primarily on cultivation techniques, over 1000 bacterial species were estimated to occupy the gastrointestinal tract (Janet M *et al*., 2008). The magnitude of the diversity of bacterial species increased from 15000 to 36000 species on the basis of rRNA sequence analysis, with the development of metagenomics technology (Frank and Pace, 2008).

A total of 3.3 million non-redundant microbial genes have been found in human faecal specimens through the metagenomics of the human intestinal tract (Meta HIT) project (Qin *et al.*, 2010). The type and amount of intestinal microbiota vary throughout the gastrointestinal tract. The colon is colonised by over 160 to 500 different bacterial species, distinguished by a wide variety of microbiological characteristics (Thursby and Juge, 2017).

Given this unusual biodiversity, gut microbes are distributed primarily in only four bacterial phyla, which are *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. The phylum of *Firmicutes* is composed predominantly of gram positive, aerobic and anaerobic bacteria, while *Clostridia* strains are popular members, but there are also possibly pathogenic gram positive (streptococci, enterococci, staphylococci) members. Next, *Bacteroidetes* are gram negative bacteria that are extremely well suited to the intestinal environment. *Actinobacteria* are usually considered beneficial by gram positive bacteria, such as the *Bifidobacterium* gene and the *Proteobacteria* phylum includes gram negative bacteria, most specifically the *Enterobacteriaceae* family.

The initiation of bacterial colonisation of the previously sterile fatal gut is known to occur at birth. In addition, there is an evidence suggesting the presence of bacteria, such as the placenta, and in womb tissues *(Aagaard et al., 2014; Rodríguez et al.*, 2015). Bacterial organisms that are first acquired by birth are primarily bacteria of *Bacteroidetes*, *Bifidobacterium*, *Prevotella* and *Lactobacillus* spp. (Putignani *et al.*, 2014). During the first year of life, the diversity of intestinal microbiota is increased, and its composition almost matches that of the adult microbial profile (Palmer *et al.*, 2007). During adulthood, the composition of gut microbiota is relatively stable. However, over time, different alterations could occur that are primarily related to dietary or overall health changes may occur over time (Shen and Wong, 2016). The *Bacteroidetes* and *Firmicutes* phylum are representative bacterial organisms which are isolated from human faeces and mucosal biopsies (Eckburg *et al*., 2005b).

Human diet has significantly altered gut microbial ecology (David *et al*., 2014). High fat and protein intakes are associated with higher *Bacteroides* levels while high fibres are associated with higher *Prevotella* levels (De Filippo *et al*., 2010; Koeth *et al*., 2013; Wu *et al*., 2013). Dietary interventions are therefore a possible method for modulating intestinal microbiota and further influencing the health of the host (Dewulf *et al*., 2013; Kovatcheva-Datchary and Arora, 2013). For instance, protein has received increased attention among food nutrients because it is the primary substrate for important beneficial short-chain fatty acids (SCFAs) and harmful putrefactive metabolites (such as ammonia, amines, hydrogen sulphides, phenols, and indoles) that can be produced by gut microbiota through proteolytic fermentation and may influence host health and contribute to disease risk (Diether and Willing, 2019; Fan et al., 2015; Kim et al., 2016; Beckhit et al., 2021).

Gut microbiota exercises its impact on the host by controlling physiological, pathophysiological and immunological procedures (Clemente *et al*., 2012; Francino, 2016; Guinane and Cotter, 2013). Following the use of experimental animal models and/or human evidence, studies have shown the effect of intestinal microbiota on inflammation improvement and have highlighted its potential as a therapeutic strategy for the treatment of inflammatory conditions (Kiesler *et al*., 2015; Reyes *et al*., 2010; Van Den Elsen *et al*., 2017). Research investigating the anti-inflammatory effect of intestinal microbiota has shown positive results in the treatment of gastric ulcers (Foxx-Orenstein and Chey, 2012; Khoder *et al*., 2016) and colorectal cancer *(Ding et al*., 2018; Gagnière *et al*., 2016; Gao *et al*., 2017). At the same time, numerous