UNIVERSIDADE DE SÃO PAULO
Faculdade de Ciências Farmacêuticas
Programa de Pós-Graduação em Ciência dos Alimentos
Área de Bromatologia

Structural-dependent effects of dietary fibers in colon cancer: Focus on dietary fiber naturally changed by the papaya ripening

Samira Bernardino Ramos do Prado

Tese para obtenção do Título de Doutor
Orientador: Prof. Dr. João Paulo Fabi

São Paulo
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RESUMO

PRADO, S. B. R. **Efeitos estrutura-dependente das fibras alimentares no câncer de cólon: foco na fibra alimentar naturalmente modificada durante o amadurecimento do mamão papaia**. 2019. 327f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2019.

O consumo de fibras alimentares (FA) está relacionado com vários benefícios à saúde como a diminuição no risco do desenvolvimento de câncer de cólon. A FA não é digerida pelas enzimas digestivas do trato gastrointestinal sendo fermentada pela microbiota intestinal do cólon. Como subproduto do processo de fermentação há a liberação de ácidos graxos de cadeia curta (SCFA) - como o butirato, o propionato e o acetato. Além do processo de fermentação, a FA pode interagir diretamente com as células epiteliais do intestino, induzindo mecanismos que também podem estar relacionados com os benefícios associados ao consumo de FA. A falta de informação sobre a FA e o câncer de cólon é, em partes, devido à complexidade de ambos, tanto do câncer quanto da estrutura da FA. As FA do mamão papaia são derivadas da parede celular da fruta apresentando diferentes estruturas dependendo do ponto de amadurecimento do fruto. Esse fato ocorre, pois, durante o amadurecimento do mamão papaia, existe uma extensa hidrólise dos polissacarídeos presentes na parede celular, diminuindo rapidamente a firmeza da polpa do fruto. Devido à falta de informações sobre FA e seus efeitos benéficos à saúde humana que são dependentes da sua estrutura, bem como a possibilidade do amadurecimento do mamão papaia naturalmente modificar as FA presentes na polpa dos frutos, a presente tese teve como principais objetivos: 1) avaliar como as enzimas que degradam a parede celular do mamão papaia afetam a solubilização e o peso molecular da parede celular do fruto; 2) investigar os efeitos diretos da pectina derivada de mamões verdes e maduros em linhagens de células de câncer, na interação com a galectina-3, e em células do tipo HEK que expressam receptores de reconhecimento de padrões (RRP); 3) avaliar a fermentação colônica humana *in vitro* utilizando as FA de mamões verdes e maduros; 4) avaliar em ratos com lesões pré-neoplásicas no cólon o efeito do consumo de ração com ou sem FA de mamões papaias verdes e maduros. As endopoligalacturonases foram relacionadas como as principais enzimas que atuam solubilizando a pectina da parede celular do mamão, afetando tanto a firmeza da polpa do fruto quanto a solubilização da pectina durante o amadurecimento. De modo geral, as FA dos mamões exerceram um efeito estrutura–dependente de acordo com a maturação do fruto. Nos experimentos utilizando linhagens de células de câncer, a pectina do mamão papaia maduro apresentou efeitos mais pronunciados na indução da morte e na inibição da migração e da agregação das células, bem como ativando os RRP, como por exemplo, os receptores do tipo toll-like, além de inibir a proteína pró-metastática galectina-3. As FA dos mamões também apresentaram diferentes resultados na fermentação colônica *in vitro* quanto à utilização das FA pelas bactérias do intestino, e também no perfil de crescimento dessas bactérias. Por fim, os animais que receberam a dieta com as FA dos mamões maduros apresentaram menor incidência de focos de criptas aberrantes do que os animais que receberam as FA provenientes de mamões verdes ou de celulose (FA da ração AIN-93G). Portanto, o estudo das FA dos mamões foi efetuado tanto durante o amadurecimento dos mamões quanto dos seus efeitos biológicos *in vitro* e *in vivo*, tendo gerado resultados inéditos relacionando as alterações bioquímicas endógenas dos frutos durante o amadurecimento com os possíveis efeitos benéficos da sua ingestão para a saúde humana.

Palavras-chaves: fibra alimentar; mamão papaia; amadurecimento; parede celular, câncer de cólon; fermentação *in vitro*; foco de cripta aberrante.

ABSTRACT

PRADO, S. B. R. **Structural-dependent effects of dietary fibers in colon cancer: Focus on dietary fiber naturally changed by the papaya ripening**. 2019. 327f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2019.

Dietary fiber (DF) consumption is related with several healthy benefits such as the decreasing risk of colon cancer development. The DF is not digested by the digestive enzymes and reach to colon where is fermented by the colonic microbiota. The fermentation process releases metabolites as short chain fatty acids (SCFA) – such as butyrate, propionate and acetate. Besides the fermentation process, the DF can directly interact with intestinal epithelial cells inducing mechanism that can also be related with the associated DF consumption benefits. The lack of information regarding DF and colon cancer are due to the complexity of both the cancer and the DF structure. The papayas DF are derived from the fruit cell wall, and they are probably naturally modified during ripening through a massive polysaccharide hydrolysis, because papayas show a very fast pulp softening. Due to the lack of information about DF and their beneficial effects to human health as well as the possibility of the natural papaya ripening to modifying the DF presented in the fruit pulp, the present thesis had as the primary objectives: 1) to evaluate how the cell-wall degrading enzymes affect the fruit cell wall solubilization and molecular weight; 2) to investigate the direct effects of the papaya pectin derived from unripe to ripe papayas in cancer cell lines, in galectin-3 interaction and in HEK cells expressing pattern recognition receptors (PRR); 3) to evaluate the human colonic *in vitro* fermentation using DF from unripe and ripe papayas as substrates; 4) to conduct an *in vivo* experiment using rats with pre-neoplastic colon lesions while receiving a diet with DF from unripe and ripe papayas. The endopolygalacturonases were the main enzymes acting on the solubilizing papaya cell wall pectin affecting both the papaya firmness and pectin structure. Overall, the papayas DF showed a ripening dependent structure–effects. In the cancer cell lines experiments, the ripe papayas pectin showed a more pronounced effects in inducing cancer cell death, inhibiting cancer cells migration and aggregation, activating PRR as toll-like receptors and inhibiting the pro-metastatic protein galectin-3. The DF from papayas also showed different aspects in colonic *in vitro* fermentation regarding the DF utilization by the bacteria and the bacteria abundance profile. Lastly, the animals receiving the diet with the DF from ripe papayas had less aberrant crypt foci in colon than the animals that received the DF from unripe papayas or cellulose (AIN-93G DF). Therefore, the study of papaya DF was carried out both during papaya ripening and its biological effects *in vitro* and *in vivo*, generating unprecedented results relating the endogenous biochemical changes of the fruits during maturation with the possible beneficial effects of their ingestion for health human.

Keywords: dietary fiber; papaya, fruit cell wall; fruit ripening; colon cancer; *in vitro* fermentation, aberrant crypt foci.

TABLE OF CONTENTS

Rationale and outline of the thesis

The present thesis is divided into chapters and this topic aims to explain the rationale that guided the work. The knowledge regarding the structure of dietary fibers has been growing in the last few years, and the structures that once appear to be responsible for minor effects on human intestine are now being related to distinct structure-dependent beneficial biological effects. In the present thesis, most of the chapters aim to explore different perspectives that together help on elucidate whether changes in the structure of dietary fiber from papaya cell wall during ripening influence its biological effects, focusing mainly on the effects of these dietary fibers on colon cancer.

Little is known about the effects of ripening on the structure of the dietary fiber derived from papaya cell wall, and even less about whether the changes in the structure of dietary fiber influences it effects on health. Thus, to gain further insight into these intriguing questions, we evaluated both the physiology of papaya fruit during ripening and the biological effects of its dietary fibers obtained at different ripening time points. These biological effects comprise mainly (1) effects on cancer cell lines treated directly with dietary fibers from papaya, (2) the colonic *in vitro* fermentation of dietary fibers from papaya, and (3) the *in vivo* effects of dietary fibers from papaya in rats with colon pre-neoplastic lesions. Overall, the idea is that the data described in the thesis help on contribute to a better understanding of the structure-function relationship of dietary fibers, as well as on contributing to the development of new concepts regarding the quality of dietary fibers for consumption as dietary supplements or through introducing specific dietary fiber rich-foods into the diet.

As the understanding regarding papaya ripening is crucial for this study, in **Chapter 1** we reviewed the physiology of papaya fruit during ripening, focusing mainly on the effects of ethylene on metabolism - which is one of the main responsible for triggering changes in cell wall (dietary fiber) structure during ripening. This review describes that a massive change in the cell wall during papaya ripening occurs resulting in fruit softness, with the role of cell wall degrading enzymes during papaya ripening being not fully understood, though. Therefore, on **Chapter 2** we explored the dynamics between the expression of the main enzymes responsible for cell wall degradation during papaya ripening and the changes of dietary fiber during papaya ripening. The results of this research article suggest that polygalacturonases massively act on papaya cell wall between

two and three days after harvesting, reducing the molecular size and increasing the solubilization of less soluble pectin. As the main changes in papaya cell wall during ripening appear to occur in pectin, we focused mainly on the study of papaya pectin fraction in the following parts of this thesis.

The recent advances regarding the effects of non-digestible carbohydrates (dietary fibers) from plant-source foods and their relationship with decreased risk of colorectal cancer were reviewed in **Chapter 3**. We described the dietary fiber-related health effects that can be divided into physicochemical effects, fermentation-related effects, and direct effects. In this review, we also included data that is presented detailed on the further chapters. This review provides insights not only to a better understating of the structure-function relationship between the intake of dietary fibers and the reduction in colon cancer risk but also to point aspects for improving nutritional recommendations for dietary fiber consumption. Finally, we highlighted that much more work still needs to be done in order to relate the structure of dietary fiber with their health effects to human consumption.

In the following chapters, we focus on the characterization of the most soluble dietary fibers from unripe and ripe papaya, as well as on their direct effects on colon cancer cell lines. However, as citrus pectin is one of the most studied dietary fiber, and only appears to have pronounced effects when its chemical structure is modified by chemical, enzymatic or thermic treatments, we decided to start exploring some biological effects of citrus pectin rather than start with papaya pectin (**Chapter 4 - part I**). Although the biological effects of modified citrus pectin on colon cancer are relatively well known, there was a lack of information regarding the biological effects of the modified citrus pectin fractions with distinct molecular size. Thus, we fractionated the modified citrus pectin according to their molecular size aiming to isolate the more active fraction, and therefore fulfill this missing information on literature regarding the well-known modified citrus pectin. We observed that modified citrus pectin fractions with smaller molecular size exerted more pronounced effects in of colon cancer cells death, as well as in inhibiting their migration. In **Chapter 4 (part II)** we studied the probable similar effects showed for the modified citrus pectin but using the papaya pectin obtained at distinct ripening time points. Papaya pectin fractions had different effects on cancer cell lines, which appear to be a structure-dependent effect. Papaya pectin from ripe (but not very ripe) showed the most pronounced effects on inducing the necroptosis of colon cancer cells and on inhibiting cancer cells migration. Besides not directly compared, the abovementioned results

suggested that while the modified citrus pectin must undergo a thermal treatment to obtain a more functional structure, the pectin from papaya could be naturally modified by cell wall degrading enzymes during the fruit ripening process. As we observed structural-dependent effects of papaya pectin at different ripening time points, we further explored the interaction between these pectin fractions and some cell receptors. The pectin interaction with the intestinal epithelial cells can be through several mechanisms, and as the intestinal cells express pattern recognition receptors, we investigated the interaction with both toll-like receptors (TLR) and nucleotide binding oligomerization domain (NOD)-like receptors. In **Chapter 5** we described the results that demonstrated the ability of papaya pectin to interact with TLR and NOD-like receptors. The activation of some TLR were observed only for the pectin fractions from ripe papayas, whereas the pectin fractions from unripe papayas appear to inhibit the activation of some TLR. At this point, the cell wall modification during papaya ripening lead to the formation of pectin fractions with unique polysaccharides structures, which were derived from less soluble pectin fraction (chelate-soluble) during papaya ripening, as proposed in **Chapter 2**. Since the whole fraction of the plant cell wall is ingested with the fruit, the next question arose was if the chelate-soluble fraction of papaya pectin could also exert different biological effects. This is because the less soluble pectin structures, the ones chelated with calcium through the "egg-boxes" structures, could be release during the digestion process. Therefore, on **Chapter 6** we investigated whether the chelate-soluble pectin (less soluble in water) had effects in cancer cell lines or whether the chelate-soluble could interact with the pro-metastatic protein galectin-3. Confirming our hypothesis, the chelate-soluble pectin extracted from the intermediate ripening point of papaya inhibited colon cancer cell lines proliferation and interacted with galectin-3. This data supports our previous results, which showed that differences in pectin structure resulting from the ripening process influence their direct effects in cancer cells.

In the last two chapters, we used the total cell wall extract (soluble and less soluble total dietary fiber) from unripe and ripe papayas to mimic the consumption and digestion of the whole dietary fiber from papayas instead of a specific fraction. In **Chapter 7** we presented the results from *in vitro* human colonic fermentation using the dietary fibers from unripe and ripe papaya. Besides the production of short chain fatty acids were similar for dietary fiber from both unripe and ripe, they were higher than apple pectin and lactulose (two fermentable polysaccharides used as standards). The bacteria utilization of the papayas dietary fiber within distinct ripening point was different, and the bacteria profile

also had some distinctions after fermentation between unripe and ripe fibers. The last part of this thesis, **Chapter 8**, brings out one of the remaining questions: whether the dietary fibers from the unripe and ripe papaya differently affect the development of pre-neoplastic colon cancer lesions. In this study, rats were chemically induced with azoxymethane to develop pre-neoplastic lesions in the colon and were then feed with dietary fiber from unripe or ripe papaya. The main results were measured by counting the aberrant crypt foci, and the dietary fiber from ripe papaya seemed to inhibit the lesions, especially the ones closer to the proximal part of the colon (closer to the cecum).

Therefore, this study contributed to the lack of information regarding the structure-function relationship between dietary fiber derived from plant sources and their biological effects. More information about the general conclusions and perspectives are available at the end of the thesis, in the 'Concluding remarks and perspectives' topic.

Chapter 1

Fast and Furious: Ethylene-Triggered Changes in the Metabolism of Papaya Fruit during Ripening

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Abstract

Papaya is a climacteric fleshy fruit characterized by a fast ripening after harvest. During the relatively short postharvest period, papaya fruit undergoes several changes in primary metabolism that result in pulp softening and sweetening, as well as the development of a characteristic aroma. Since papaya is one of the most cultivated and appreciated tropical fruit crops worldwide, extensive research has been conducted to not only understand the formation of the quality and nutritional attributes of ripe fruit but also to develop methods for controlling the ripening process. However, most strategies to postpone papaya ripening, and therefore to increase shelf life, have failed to maintain fruit quality. Ethylene blockage precludes carotenoid biosynthesis, while cold storage can induce chilling injury and negatively affect the volatile profile of papaya. As a climacteric fruit, the fast ripening of papaya is triggered by ethylene biosynthesis. The generation of the climacteric ethylene positive feedback loop is elicited by the expression of a specific transcription factor that leads to an up-regulation of 1-*aminocyclopropane-1-carboxylic acid* (ACC) *synthase* (ACS) and *ACC-oxidase* (*ACO*) expression, triggering the system II ethylene biosynthesis. The ethylene burst occurs about three to four days after harvest and induces pectinase expression. The disassembling of the papaya cell wall appears to help in fruit sweetness, while glucose and fructose are also produced by acidic invertases. The increase in ethylene production also results in carotenoid accumulation due to the induction of cyclases and hydroxylases, leading to yellow and red/orange colored pulp phenotypes. Moreover, the production of volatile terpene linalool, an important biological marker for papaya sensorial quality, is also induced by ethylene. All these mentioned processes are related to papaya sensorial and nutritional quality. We describe the understanding of ethylene-triggered events that influence papaya quality and nutritional traits, as those characteristics are a consequence of an accelerated primary metabolism during fruit ripening.

Key-words: papaya; climacteric fruits; ethylene; primary metabolism; cell wall; sugars; carotenoids, volatiles.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; AI, acid invertase; ACO, 1-aminocyclopropane-1-carboxylic acid oxidase; ACS, 1-amino cyclopropane-1-carboxylic acid synthase; BFF, β-fructofuranosidase; ZDS, ζ-carotene desaturase; CHYB, carotene hydroxylases; GC-O, chromatography-olfactometry; ERFs, ethylene response factors; GalA, galacturonic acid; GPP, geranyl diphosphate; Glc, glucose; HG, homogalacturonan; LIS, linalool synthase; LCY-β, lycopene β-cyclases; 1-MCP, 1-methylcyclopropene; MEP, 2-C-methyl-D-erythritol 4-phosphate; PL, pectate lyases; PME, pectin methyl esterases; PG, polygalacturonases; PDS, phytoene desaturase; RG-I, rhamnogalacturonan type I; RG-II, Rhamnogalacturonan type II; SAM, S-adenosyl methionine; SPS, Sucrose phosphate synthase; STP, sugar transporter; SS, sucrose synthase; UDP-Glc, diphosphate glucose; UTR3, UDP-galactose transporter 3; XG, xylogalacturonans; XYL, xyloglucan; Xyl, xylose.

Introduction

Papaya (*Carica papaya* L.) is a typical climacteric fleshy fruit that is appreciated worldwide because of the sweetness and characteristic flavor of its soft yellow or orange/red pulp [1,2]. Tropical countries from Asia are the main producers of papaya, accounting for 56% of worldwide production. However, countries from South America (16%), Africa (10%), and Central America (9%) are also important producers of papaya (Food and agriculture Organization of the United Nations [FAOSTAT], 2017). As papayas have a relatively short shelf life compared to other fruits, maintaining fruit quality during transport from producing countries to consumer centers (e.g., USA and Europe) is a challenge. In 2016 the main countries that produced papayas for exportation were Mexico (47%), Guatemala (14%), and Brazil (11%; FAOSTAT, 2017), with Mexico being the main supplier to the United States and Brazil the main supplier to Europe [5].

European recommendations for papaya exporting countries take into account fruit softening as a determinant factor in fruit shelf life [6], since the fast softening during papaya ripening facilitates physical injury during handling and transportation. Thus, as the susceptibility of papayas to disease increases proportionally with softening [7], the recommendation for exportation is to maintain the fruit at 10 ºC during shipping to prevent overripening due to heat [6]. However, as will be discussed later, low temperatures negatively impact some fruit quality attributes of ripe papayas.

The ripening of fleshy fruits is a physiological process that alters appearance, texture, flavor, and aroma. These changes function to attract seed-dispersing organisms [8]. In climacteric fruit, such as tomatoes, bananas, and papayas, the onset of ripening coincides with an increase in respiration and ethylene production, the latter being essential to induce molecular mechanisms responsible for accelerating senescence and for the physiological changes that occur during ripening [9,10]. The ripening process in climacteric fruits induces changes in both sensorial and nutritional qualities that are essential for consumer acceptability. Some climacteric fruits are harvested unripe and treated with exogenous ethylene or ethylene-derived molecules to precipitate ripening. Thus, ethylene appears to be the main hormone responsible for regulating the molecular pathways that influence the development of the sensorial and nutritional attributes of climacteric fruits [11]. It has long been known that the safe and effective control of ethylene-mediated responses could extend

the postharvest shelf life of climacteric fruits [12]. However, interfering with natural ethylene-mediated responses during ripening could also negatively impact fruit quality.

While the mechanism by which ethylene is involved in fruit ripening has been thoroughly studied, efforts are still needed to fully understand this process. The ethylene burst in climacteric fruit is controlled by an autocatalytic mechanism, named system II, that synthesizes ethylene [13,14]. Ethylene synthesis involves the conversion of S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by the action of 1-amino cyclopropane-1-carboxylic acid synthase (ACS), in which ACC is converted to ethylene by ACC oxidase (ACO) [15]. ACS and ACO enzymes have already been identified in papayas, and their responses are increased with ethylene production and reduced when ethylene is blocked [16]. A decrease in ACS and ACO occurs in papayas stored at low temperatures, but levels are restored after exogenous ethylene treatment [17]. The ethylene downstream cascade involves multiple transcription factors, including ethylene response factors (ERFs), that are involved in the control of plant growth, defense, responses to the environment, and plant hormones [18], including those involved in the papaya ripening process [19]. Transcription factors of the MADS-box, NAC and AP2/ERF gene families are also involved in the control of papaya ripening [20]. More recently a NAC transcription factor, rather than MADS transcription factors, was found to regulate ACS and ACO expression during papaya ripening [11]. Papaya has not undergone whole-genome duplication, unlike other climacteric fruits where this process has been utilized to duplicate the MADS transcription factors that form the ripening circuits [21,22]. NAC is one of the largest plant-specific transcription factor families, with members involved in many developmental processes such as senescence, stress, cell wall formation, and embryo development [11]. Lü et al. (2018) have suggested that instead of neofunctionalization of the duplicated MADS genes, plants without whole genome duplication may have repurposed their carpel senescence NAC to generate a positive feedback loop where ethylene regulates ripening, as is the case with papayas. They also suggested that ethylene generated by this feedback loop is autocatalytic. A NAC transcription factor expressed in climacteric fruits, such as papayas and peaches, binds to the promoter regions of some of the key ripening-related genes stimulating their expression in pigment accumulation, volatile secondary metabolite production, cell wall softening, and sugar accumulation [11].

Therefore, the ethylene-mediated effects in fruit metabolism that influence the softening, sweetness, flavor, and color of papaya pulp during ripening will be further discussed.

1. Pulp softening is the main biochemical modification that occurs during papaya ripening

In climacteric fleshy fruits, researches and producers give special attention to ethylene-induced textural changes during ripening, as changes in peel and pulp influence softening, crispness, and juiciness [23] but also increase postharvest losses [7]. In fact, textural changes in most of the fleshy fruits result from complex mechanisms that primarily influence plant cell wall architecture, whose breakdown is considered as the major factor responsible for the pulp softening process [24].

The cell wall architecture of fleshy fruits is comprised of complex polysaccharides, such as pectin, hemicellulose, and cellulose, as well as minor components including proteins and phenolic compounds [25]. Cellulose is comprised of long, rigid, and inextensible microfibrils of 1,4-β-D-glucose (Glc) residues, which are bound tightly together by hydrogen bonds [24]. Hemicelluloses represent a diverse range of structural polymers that constitute the plant cell wall within fruit pulp [26]. In dicotyledonous plants, such as papayas, xyloglucan (XYL) is the major hemicellulose [27]. As with cellulose, XYL consists of a backbone of 1,4-β-D-Glc residues such as cellulose, but smaller and substituted with 1-6-α-D-xylose (Xyl) side chains. Furthermore, these Xyl side chains can be substituted at the O-2 position with β-galactose (Gal) or α-arabionse (Ara; Scheller and Ulvskov, 2010). Pectin is a complex and heterogeneous polysaccharide that is mainly comprised of α-1,4-D-galacturonic acid (GalA) residues that have varying degrees of acetyl and methyl esterification, and these residues are called homogalacturonan (HG). Xylosylation may further modify HG into xylogalacturonans (XG). Pectin also contains structures made up of repeating units of intercalated GalA (1,4-α-D-GalpA) and rhamnose (1,2-α-L-Rhap) called rhamnogalacturonan type I (RG-I). These structures have side groups of arabinose (arabinan), galactose (galactan), and type I arabinogalactan at the O-4 position of the Rha residues [28,29]. Rhamnogalacturonan type II (RG-II) structures are less common in papayas and are composed of HG molecules with side groups of up to 13 different sugars and more than 20 types of glycosidic linkages [28,30]. The firmness of fleshy fruits results from turgor pressure maintenance by the cell wall while also

maintaining cellular adhesion [31]. Pulp softening occurs by the water dissolution of the majority of these polysaccharides from the primary cell wall and middle lamella, with pectin being the main one [32].

Structural changes that occur in the cell wall during ripening are regulated by hydrolases responsible for degrading cell wall polysaccharides [9,33], whose expression is generally regulated by ethylene production [27]. Fruit softening is a complex event that involves several enzymes including pectinases and hemicellulases; however, pectinases, such as polygalacturonases (PGs), pectate lyases (PLs), and pectin methyl esterases (PMEs) appear to be the major enzymes that act on fleshy fruit softening. Polygalacturonases remove the galacturosyl residues from pectin [34], PLs cleave de-esterified pectin [35], and PMEs hydrolyze methyl-groups of esterified polyuronides [36]. Furthermore, side chains of pectin can be degraded by other glycosidases, such as β-galactosidases, which remove the galactosyl residues from pectin and from XYL [37]; α-arabinofuranosidases, which remove arabinosyl from pectin [38,39]; and rhamnogalacturonases, which remove α-1,2 linkages between galacturonosyl and rhamnosyl residues [40].

Despite multiple glycoside hydrolases seeming to be responsible for papaya softening, the main enzymes that play a central role in pulp softening are the PGs [41]. Some contribution of hemicellulose degradation to pulp softening appears to occur as an increase in endoxylanase expression occurs during papaya ripening [42]. Furthermore, β-galactanases are also related to papaya pulp softening through the hydrolysis of both the pectic and the hemicellulosic fractions [41,43]. In order to understand the role of ethylene in the expression of cell wall-degrading enzymes, researchers have treated papayas with 1-methylcyclopropene (1-MCP), an ethylene antagonist. As expected, this had a strong effect on pulp softening [1]. The pulp firmness of 1-MCP-treated papayas decreased marginally during ripening, although not enough to reach an edible state, and there was no detectable PG activity. Notably, 1-MCP-treated papayas were unable to soften at the same rate as untreated papayas [1,44,45]. Treatment with 1-MCP also reduced endoxylanase protein levels [42].

To confirm that ethylene affects PG activity and, therefore, pulp softening during papaya ripening, Fabi et al. (2009) found that treatment with exogenous ethylene had induced PGs expression with a concomitant increase in pulp softening. Furthermore, agroinfiltration of PG1 in 1-MCP-treated papayas significantly enhanced pulp softening compared with 1-MCP-treated papayas that were agroinfiltrated with an empty vector [41].

Papaya cell wall structural changes during ripening involve pectin with the solubilization of long chains of galacturonans and a decrease in the molecular weight of polysaccharides [7,46,47]. Polygalacturonases act on papaya pulp softening by mobilizing high molecular weight pectin from less soluble to more soluble cell wall fractions, especially pectin that is tightly bound to cellulose/hemicellulose, and pectin that are bound to each other by calcium bridges [48]. Furthermore, the degree of methyl esterification in papaya pectin changes during ripening since unripe papaya pectin has a lower degree of methyl esterification compared to ripe papaya pectin [7,49–51]. This variation during papaya ripening was firstly associated to higher PME activity [7]. However, no increase in gene expression of PME appears to occur during papaya ripening [20,41,48,52], and the activity of PG does not require the simultaneous removal of methyl-esterified groups from pectin [41]. Therefore, recent studies support the hypothesis that the increase in the degree of methyl esterification during papaya ripening is a result of the enrichment of the water-soluble pectin fraction that comes from the insoluble fraction due to the massive action of PG rather than an association with increased PME activity (**Figure 1**; Fabi et al., 2014; Prado et al., 2016, 2017). The resulted high methylated low-molecular pectin found in ripe papayas showed anticancer effects in diverse *in vitro* tests [49,50].

Although the use of MCP-1 is useful in gaining further insight into the role of ethylene in papaya softening, cold storage is another way to decrease ethylene action after harvesting. This latter approach is useful as a postharvest technique as it decreases fruit ripening rates and, therefore, pulp softening [53]. The storage of 'Golden' papaya at 10 °C for 10 days had been found to be effective in reducing ethylene production and fruit ripening. Notably, after a 10-day cold storage, fruits can be stored at room temperature to restore ethylene production and pulp softening [53]. However, when cold storage occurs for a longer period (e.g., 20 days at 11 °C), ethylene production did not recover when the fruit was subsequently stored at ambient temperatures [54]. It seems that the prolonged inhibition of ethylene, either by the inhibition of receptor sites (1-MCP) or by prolonged

storage at low temperatures, strongly affects the recovery of the ethylene-mediated response, which negatively influences the pulp softening that is crucial to the quality of the ripe fruit.

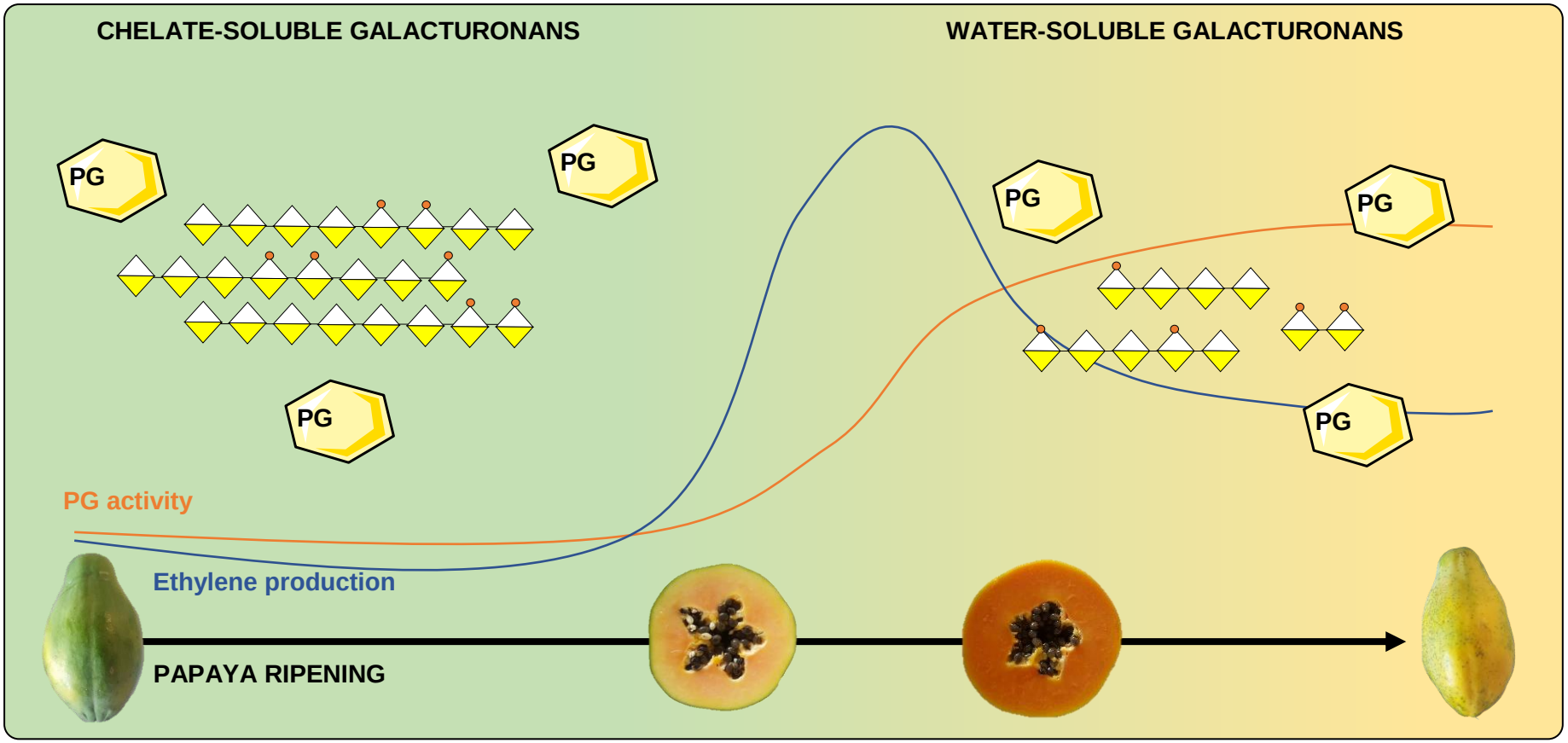

Figure 1. Ethylene production and PG activity during papaya ripening: papaya pectin cell wall solubilization. Ethylene triggers PGs that massively solubilize high molecular weight pectin by action in the non-methylated areas and releasing the low molecular weight fractions that will be enriched in methylated fractions due to the lower activity of PME in ripe papayas. PG: polygalacturonase.

2. Pulp sweetness as a result of papaya primary metabolism

The qualitative and quantitative composition of primary soluble sugars is crucial to papaya sweetness, although fruit firmness also plays a role as there is a correlation between pulp softening and the perception of sweetness during consumption [55]. Thus, it is necessary to understand the key regulatory enzymes involved in the metabolism of soluble sugars, as well as the endogenous and exogenous factors that influence these biochemical pathways, so as to improve both preharvest management and postharvest handling to increase the final sensorial quality of ripe papayas. In papayas, the increment in soluble sugars occurs mainly during fruit growth while still attached to the plant [56].

In most fleshy fruits, there are three main enzymes that have a key regulatory role in the accumulation of soluble sugars: Sucrose phosphate synthase (SPS), sucrose synthase (SS), and acid invertase (AI; Zhou and Paull, 2001). In papayas, sugar accumulation begins after seed maturation and is accompanied by increased activity of SS during fruit development. Acid invertase also appears to increase throughout papaya development [56], and its expression is reduced in harvested unripe papayas. Another increase in AI expression has also been observed after the onset of ethylene production during ripening [57]. Sucrose phosphate synthase activity remains low throughout papaya development however [56]. After harvesting, SPS activity follows the tendency of sucrose formation, since the ratio between SPS activity and sucrose content is constant throughout the papaya ripening process [55]. SPS is a highly conserved glycosyltransferase in dicots that catalyzes the transfer of glucose from uridine diphosphate glucose (UDP-Glc) to D-fructose-6-phosphate, thereby forming D-sucrose-6-phosphate [58]. As SPS also catalyzes the reversible reaction, it is considered as a key control point of sucrose biosynthesis in both monocots and dicots [59]. Sucrose synthase is also a glycosyltransferase, but it catalyzes the reversible formation of UDP-Glc and D-fructose from UDP and D-sucrose [60]. Although SS could act in Glc linked to other nucleotide diphosphate sugars than UDP, such as adenosine diphosphate glucose (ADP-Glc), UDP is the preferred substrate in plants [61]. Finally, AI can control the balance between sucrose, glucose and fructose in fleshy climacteric fruits by an irreversible reaction that cleaves sucrose [62].

Climacteric fruits, such as bananas, commonly increase soluble sugars content after harvesting through starch degradation, which directly correlates with pulp sweetening (Shiga et al., 2011; Aquino et al., 2016). Since unripe papayas have low starch content (less

than 3% by fresh weight; Oboh et al., 2015), most of the soluble sugars in papayas accumulate during fruit development. However, there is also an increase in sucrose, glucose, and fructose, as well as a pattern of expression and activity of both AI and SPS during ripening [55,57]. These results suggest a possible role for ethylene-mediated effects on soluble sugar accumulation in ripe papayas. This hypothesis was confirmed by a previous study of our group [1], which demonstrated that 1-MCP-treated papaya have a distinct pattern of sucrose synthesis during ripening compared to untreated papayas. More recently, Shen et al. (2017) showed that other genes related to soluble sugar metabolism, including *UDP-galactose transporter 3* (*UTR3*), *sugar transporter* (*STP*), and *β-fructofuranosidase* (*BFF*), were induced during the ripening of ethylene-treated papaya and reduced in 1-MCP-treated papaya. However, despite ethylene appearing to be important in enhancing *UTR3*, *STP*, and *BFF* expression, it is unknown whether ethylene-induced changes in the expression pattern of these enzymes affect soluble sugar metabolism during ripening and, therefore, the sensorial quality of papaya.

During papaya ripening, the sucrose content appears to reduce after the onset of ethylene production, which is in agreement with the increase in AI expression [55,67]. In contrast, 1-MCP-treated papayas have been found to have a 10-fold higher level of sucrose compared to untreated ripe fruit [1]. Thus, as AI activity appears to be strongly regulated by ethylene during papaya ripening (**Figure 2**), exogenous treatments or conditions that affect ethylene production may affect the ratios between sucrose, glucose and fructose, thereby influencing pulp sweetness.

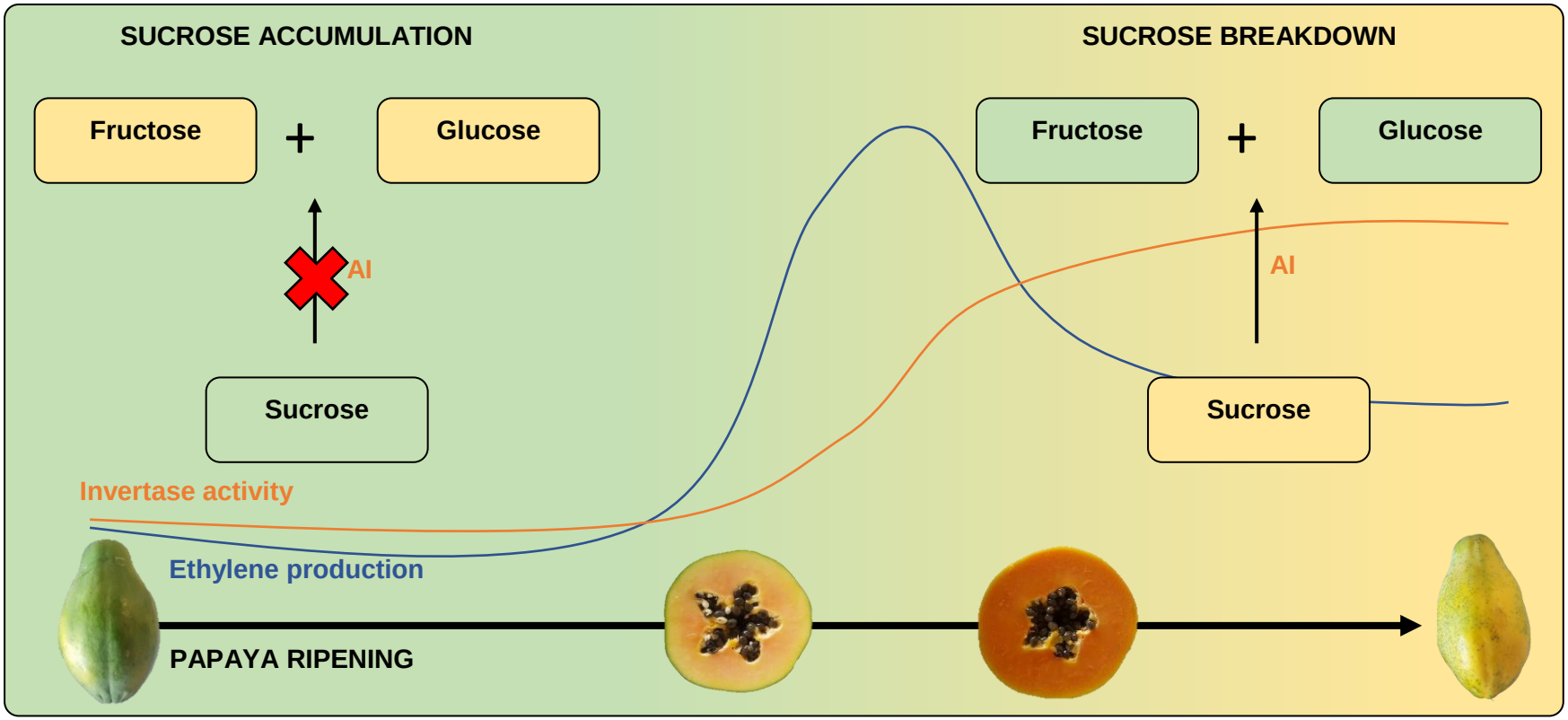

Figure 2. Ethylene production and invertase activity during papaya ripening: papaya sucrose breakdown. Invertase activity is regulated by ethylene burst since sucrose is higher in unripe papayas or in papayas that ethylene perception were blocked, with a subsequent increase in fructose and glucose after ripening/ethylene production. AI: acid invertase.

The use of gamma irradiation in fleshly fruits such as guavas [68] and tomatoes [69] could represent an effective method for fruit decontamination thus reducing postharvest losses [70]. Depending on the intensity of the applied gamma irradiation, the sensorial quality of fruits could be negatively affected because of irradiation-induced changes in fruit metabolism. In papaya, the application of standard irradiation intensities between 0.5 and 1.0 kGy in unripe fruit did not appear to negatively influence fruit ripening [71]. However, analysis of fruit metabolism revealed that these gamma irradiation intensities could reduce soluble sugars content in ripe papayas. This reduction appears to be related to a decrease in AI activity, and these changes are associated with reduced ethylene production throughout the ripening of the irradiated fruit [57].

In addition to gamma irradiation, ozone application has been proposed as a method for fruit decontamination. Furthermore, ozone treatment is used to extend shelf life by reducing oxygen concentrations during fruit storage and shipping, thereby delaying the ripening of climacteric fruits [72]. Thus, as ozone influences fruit respiration and therefore the onset of ethylene production in climacteric fruits, it is expected that this postharvest treatment will also affect soluble sugar metabolism during papaya ripening. Although a previous study did not report significant differences between the total soluble solid content of ozone-treated and untreated papayas [73], the soluble sugars ratio between sucrose and glucose/fructose in ripe fruits could be altered. Recently, the treatment of unripe papaya with plant extracts, such as Neem (*Azadirachta indica* Juss), has been proposed as an alternative for maintaining food quality for a longer postharvest period [74]. However, as with ozone treatment, the observation of fruit quality maintenance for a longer period was not accompanied by an evaluation of soluble sugar metabolism. Therefore, further studies are needed to confirm the effects of ozone as well as other postharvest treatments that may affect ethylene production, since there is a clear role of ethylene on enzymes that orchestrate the metabolism of soluble sugars during papaya ripening.

3. Climacteric alteration of papaya flavor

Papayas have a characteristic sweet flavor that has been studied for more than half a century [75,76]. The volatile profile of papaya consists of a mixture of compounds including esters, terpenes, alcohols, and ketones [77–81]. Although there is great heterogeneity among the volatile profiles of distinct papaya varieties [78,80,82], some compounds appear to be characteristic of the papaya aroma. In this context, linalool and

their oxidative derivatives are generally regarded as the main volatile compounds in most of the distinct cultivars of papaya [53,83,84] along with low molecular weight esters, including ethyl butanoate and methyl butanoate [77,85,86]. Considering that the increase in volatile esters is significantly higher in harvested papayas compared to fruit that is still attached to the plant [81], and considering the magnitude of difference between the volatile profiles of unripe and ripe papayas [53,81], it appears that ethylene plays an important role in the development of flavor during papaya ripening.

Balbontín et al. (2007) suggested that most of the volatile esters synthesized during papaya ripening are derived from primary metabolism compounds, such as fatty acids and amino acid. The release of these compounds is stimulated by ethylene treatment [87,88]. Ethyl acetate, ethyl octanoate, and methyl hexanoate were also found to not be induced in 1-MCP-treated papayas, whereas ethylene-induced papayas increased the amounts of these volatile esters throughout ripening [86]. Interestingly, volatile esters with a higher molecular weight, including butyl hexanoate and octyl acetate, reached higher values in 1-MCP-treated papayas compared to both untreated and ethylene-treated papayas. These results suggests that only the synthesis of the main esters related to aroma quality in ripe papaya—which are those volatile compounds with lower molecular weight produced from C1 and C2 alcohols and C6 and C8 acyl-coenzyme A—were enhanced during the onset of ethylene production [86].

The volatile profile of ripe papayas also consists of branched-chain volatiles [84,89] derived mainly from the amino acid precursors isoleucine and valine, which are responsible for the formation of ethyl-2-methyl and butyl-2-methyl esters. The synthesis of these branched-chain volatiles also appears to be regulated by ethylene, as 1-MCP treated papayas have reduced ethyl-2-methyl butanoate levels [86].

The abovementioned results regarding the synthesis of volatile compounds during ripening provide insights into the development of aroma in ripe papayas. However, little is known about the relationships among the metabolism of these volatile compounds and the sensorial quality of the ripe fruit. In this context, a recent study applied a gas chromatography-olfactometry (GC-O) assisted approach to optimize the extraction and detection of the main volatile compounds responsible for the aroma of ripe papayas [89]. In GC-O a panel of human assessors describes the aroma of each of the volatile compounds from a sample that has been previously separated through gas chromatography, allowing

the identification of the main peaks responsible for the overall aroma of the sample [90]. In summary, GC-O refers to the use of human assessors as a sensitive and selective detector of odor-active compounds [91], and it is a useful tool to assess the contribution of each volatile compound to a fruit's aroma. Studies have successfully applied GC-O-assisted approaches or aroma dilution analysis to assess the volatile profile of papayas [77,89,92]. Jirovetz et al. (2003) and Pino (2014) found linalool as the major compound in papaya flavor. However, the major compounds considered as odor-active and contributors to the typical papaya aroma found in other studies were δ-octalactone (sweet and herbal), benzyl isothiocyanate (papaya), methyl butanoate (fruity), and ethyl butanoate (fruity; Pino 2014; Rocha et al., 2017).

Gomes et al. (2016) explored the volatile profile of papayas in response to cold storage, which clearly affects ethylene production [54]. The authors explored if the cold storage of papayas at temperatures in which the fruit is resistant to cold injury influenced the volatile profile in ripe papayas. The authors found that when papayas were left at 10 °C for 10 days and then subsequently at ambient temperature to complete the ripening process, the fruits were able to restore ethylene production, as well as the development of the loss of green color and the increase in pulp softening to a similar extent to that of fruit stored at ambient temperature, but the process was postponed by a few days. However, there were striking differences between the volatile profiles of the two groups. Interestingly, the synthesis of linalool, regarded in GC-O as one of the main volatile compounds in papaya, was affected by cold storage. These reduced linalool levels in cold-stored papayas appeared to be related to the downregulation of *linalool synthase* (*LIS*) expression [53]. Façanha (2016) also found reduced levels of linalool throughout the ripening of 1-MCP-treated papayas and increased levels of this volatile compound in ethylene-treated papayas. Thus, as LIS uses geranyl diphosphate (GPP) to synthesize linalool in a single step reaction [94], the reduced *LIS* expression, and therefore reduced levels of linalool in both cold storage papayas and in 1-MCP-treated papayas, strongly suggests a possible role of ethylene in linalool biosynthesis through modulation of *LIS* expression.

GPP originates from the plastid-localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which is important not only in the biosynthesis of linalool and other volatile compounds, including β-ionone and 6-methyl-5-hepten-2-one, but also in

carotenoid biosynthesis and in the development of the characteristic of pulp color in ripe papayas.

4. Pulp color changes in ripening papayas as a consequence of carotenoid synthesis

The characteristic color of ripe papaya pulp (yellow or orange/red) is due to different types of carotenoids. Carotenoids are molecules with a general structure that consists of a 40-carbon acyclic polyene chain containing 9–11 conjugated double bonds and with or without terminating rings, and they are classified as carotenes (hydrocarbons) or as xanthophylls (oxygenated derivatives; Khoo et al., 2011). Distinct papaya varieties have different pulp colors depending mainly on their carotenoid metabolism during ripening. In general, orange/red varieties have relatively high amounts of lycopene, which is a central compound in the metabolism of carotenoids during papaya ripening and is responsible for the red color not only in papayas [96] but also in tomatoes [97], guavas [98], and watermelons [99].

Most of the over 600 naturally occurring carotenoids [100] originate from the MEP pathway (**Figure 3A**), which starts with a reaction between pyruvate and glyceraldehyde-3-phosphate, resulting in the downstream production of isopentenyl diphosphate (IPP) and dimethyl allyl diphosphate (DMAPP; Ruiz-Sola and Rodríguez-Concepción, 2012; Yang and Guo, 2014). Then, three IPP molecules and one DMAPP molecule are used as substrates by geranyl-geranyl diphosphate (GGPP) synthase for the synthesis of GGPP, a 20-carbon molecule [103]. In addition to the presence of relatively high levels of lycopene, orange/red papayas present lower amounts of carotenoids that are synthesized downstream to lycopene in the MEP pathway, such as β-carotene, β-cryptoxanthin, and zeaxanthin [104]. For both papaya cultivars ‘Golden’ and ‘Sunrise Solo’, all-trans-lycopene was the main carotenoid in early stages and all-trans-β-cryptoxanthin was the main carotenoid in overripe fruits [105].

Yellow pulp varieties are characterized by the presence of these last carotenoids with very low to no detectable levels of lycopene [106]. As the metabolism of papaya carotenoids starts from phytoene and occurs in a well-known cascade process (**Figure 3B**), it is possible to establish a relationship between the pattern of enzymes that acts downstream to phytoene and the color of papaya pulp during ripening. Geranyl-geranyl

diphosphate is the precursor of chlorophylls, ubiquinones, and tocopherols. Phytoene synthase (PSY) uses two molecules of GGPP to produce phytoene, a colorless 40-carbon acyclic polyene molecule, which is the first step in carotenoid biosynthesis in the MEP pathway. Phytoene can be further used as a substrate by phytoene desaturase (PDS) to produce ζ-carotene, which can be a substrate for ζ-carotene desaturase (ZDS) for the synthesis of lycopene, a bright red carotenoid widely found in the pulp of orange/red papaya [107]. In yellow papayas there is no significant accumulation of lycopene because of the conversion of phytoene by PDS and ZDS and by both lycopene β-cyclases (LCY-β) and carotene hydroxylases (CHYB). These enzymes rapidly convert lycopene into xanthophylls and β-carotene [106,108]. In orange/red papayas, the initial stages of ripening are characterized mainly by the presence of xanthophylls, including β-cryptoxanthin, which are synthesized from lycopene downstream by lycopene β-cyclase (LCY-β; Blas et al., 2010; Schweiggert et al., 2011). However, after the onset of ethylene production in red/orange papayas, the conversion of lycopene into cyclic carotenoids appears to be strongly decreased due to lycopene accumulation in pulp [96,106]. The accumulation of lycopene in orange/red papayas compared to yellow papayas seems to occur both by a frame shift mutation in the *LCY-β2* gene, which results in a dysfunctional enzyme phenotype, and by other LCY genes (e.g., *LCY-β* and *LCY-ε*) that are downregulated during orange/red papaya ripening [66]. The ζ-carotene desaturase enzyme responsible for converting phytoene into lycopene shows a different pattern of expression during ripening and also between the cultivars ‘Golden’ and ‘Sunrise Solo’, while the lycopene β-cyclase gene, responsible for converting lycopene to β-carotene, is up-regulated in both cultivars [105].

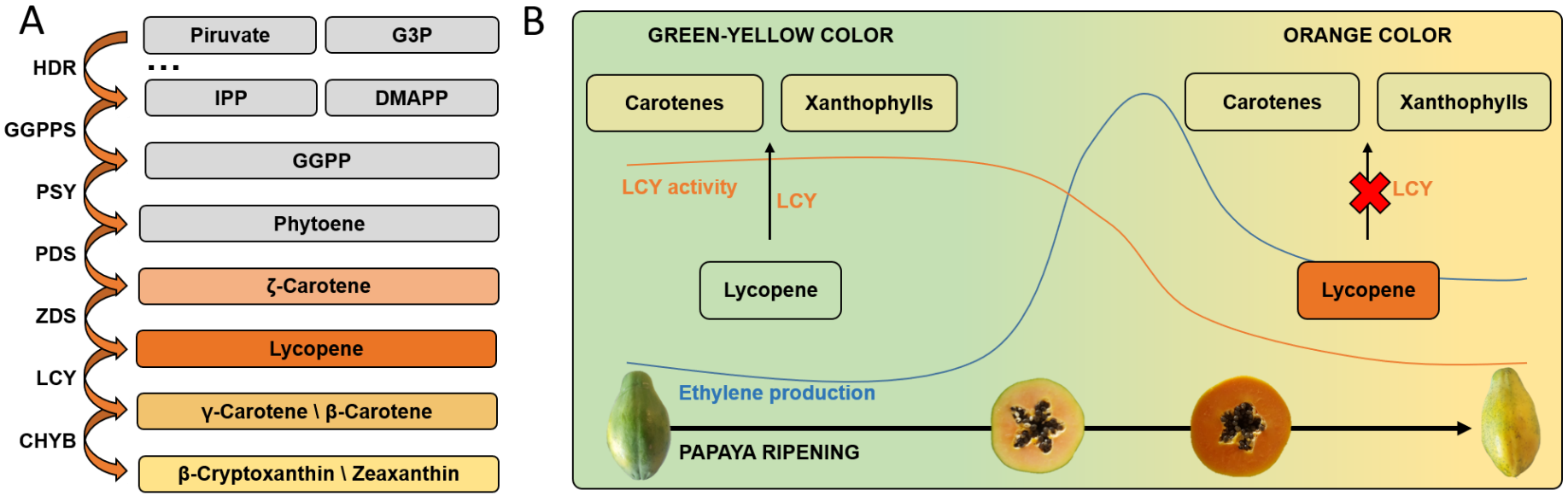

Figure 3. Ethylene production and carotenoids accumulation (LCY activity) during papaya ripening: papayas green/yellow color changing to orange/red color. A) Carotenoids derivated from MEP pathway. B) Papaya LCY activity during ripening drives the lycopene accumulation and pulp color changes through the decreased conversion of lycopene in carotenes and xanthoplylls. G3P: glyceraldehyde-3-phosphate; IPP: isopentenyl diphosphate; DMAPP: dimethyl allyl diphosphate; GGPP: geranyl-geranyl diphosphate; HDR: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; GGPPS: geranyl-geranyl diphosphate synthase; PSY: Phytoene synthase; PDS: phytoene desaturase; ZDS: ζ-carotene desaturase; LCY: lycopene cyclase; CHYB: carotene hydroxylase.

Interestingly, both ethylene- and 1-MCP-treated papayas had lower levels of minor carotenoids as compared to those of untreated papaya, similarly to what was previously reported for the major carotenoids [1,96]. Furthermore, the treatment of distinct papaya varieties with 1-MCP significantly reduced the carotenoid content in fruit pulp throughout ripening [1,96,109]. Barreto et al. (2011) suggested that the impairment on carotenoid accumulation in papaya pulp by 1-MCP could occur either by the consumption of early carotenoid precursors including GGPP, or by inhibiting PSY or PDS activity. The latter hypothesis was confirmed by Fu et al. (2016), who revealed that a transcription factor (CpNAC1) induced by ethylene enhances the expression of PDS genes (e.g., *CpPDS2* and *CpPDS4*). Recently, Fu et al. (2017) provided new insights into the role of other transcription factors that regulate ethylene responses and are involved in the regulation of several genes related to carotenoid biosynthesis. Therefore, as with pulp softening, sweetness, and the development of flavor, the carotenoid content in papayas is also regulated by ethylene-mediated responses during fruit ripening. Thus, while further studies are needed to define the specific genes whose expression relates to changes in the carotenoid content in papaya pulp, it is known that the reduction of ethylene production at low temperatures influences the composition of carotenoids in ripe papaya pulp [112].

5. Conclusions

Changes in the primary metabolism of papaya are mainly dependent on ethylene, whose onset burst occurs two to three days after the harvest of unripe fruit. Ethylene-triggered events during papaya ripening include an increase in PG and AI expression that are related to pulp softening and sweetening, respectively, as well as changes in carotenoid metabolism that influence both aroma and color, thereby leading to the formation of the expected quality attributes in ripe papaya. As ethylene-triggered events clearly affect the final quality of ripe papayas, studies have investigated the regulatory mechanisms that regulate ethylene function in papaya. Despite recent findings that highlight the ethylene-triggered events during papaya ripening, more efforts are needed to fully understand the key downstream regulators of ethylene in papaya pulp to better develop pre- and postharvest practices to extend papaya shelf life without resulting in losses in quality and nutritional aspects.

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Chapter 2

Physiological Degradation of Pectin in Papaya Cell Walls: Release of Long Chains Galacturonans Derived from Insoluble Fractions during Postharvest Fruit Ripening

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Chapter 3

Ingestion of non-digestible carbohydrates from plant-source foods and decreased risk of colorectal cancer: A review on the biological effects and the mechanisms of action

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Abstract

The hypothesis that links the increase in the intake of plant-source foods to a decrease in colorectal cancer (CRC) risk has almost fifty years. Nowadays, systematic reviews and meta-analysis of case-control and cohort studies confirmed the association between dietary patterns and CRC risk, in which the non-digestible carbohydrates (NDC) from plant-source foods are known to play beneficial effects. However, the mechanisms behind the physicochemical properties and biological effects induced by NDC on the decrease of CRC development and progression remain not fully understood. NDC from plant-source foods consist mainly of complex carbohydrates from plant cell wall including pectin and hemicellulose, which vary among foods in structure and in composition, therefore in both physicochemical properties and biological effects. In the present review, we highlighted the mechanisms and described the recent findings showing how these complex NDC from plant-source foods are related to a decrease in CRC risk through induction of both physicochemical effects in the gastrointestinal tract, fermentation-related effects, and direct effects resulting from the interaction between NDC and cellular components including toll-like receptors and galectin-3. Studies support that the definition of the structure-function relationship—especially regarding the fermentation-related effects of NDC, as well as the direct effects of these complex carbohydrates in cells—is crucial for understanding the possible NDC anticancer effects. The dietary recommendations for the intake of NDC are usually quantitative, describing a defined amount of intake per day. However, as NDC from plant-source foods can exert effects that vary widely according to the NDC structure, the dietary recommendations for the intake of NDC plant-source foods are expected to change from a quantitative to a qualitative perspective in the next few years, as occurred for lipid recommendations. Thus, further studies are necessary to define whether specific and well-characterized NDC from plant-source foods induce beneficial effects related to a decrease in CRC risk, thereby improving nutritional recommendations of healthy individuals and CRC patients.

Keywords: Colorectal cancer; Dietary fibre, Fermentation; Galectin-3, Non-digestible carbohydrates, Pattern Recognition Receptors; Pectin; Tool-like receptors.

Abbreviations: AceA, Aceric acid; Ac, Acetylated; Api, Apiose; Ara, Arabinose; CRD, Carbohydrate recognition domain; CLR, C-type lectin receptors; CRC, Colorectal cancer; DAMP, damaged-associated molecular patterns; Fuc, Fucose; Gal, Galactose; GalA, galacturonic acid; Gal-3, Galectin-3; Glc, Glucose; HSP, Heat shock proteins; HDAC, Hystone deacetylases; HG, Homogalacturonan; IEC, Intestinal ephitelial cells; LPS, Lipopolysaccharide; LRR, C-terminal leucine-rich repeat motif; Man, Mannose; MAPK, mitogen-activated protein kinase; Me, Methylated; MCP, Modified citrus pectin; NDC, Non-digestible carbohydrates; NRL, Nucleotide binding oligomerization domain (NOD)-like receptors; PAMP, Pathogen-associated molecular patterns; PRR, Pattern recognition receptors; RIPK, receptor-interacting serine/threonine-protein kinase; RG, Rhamnogalacturonan; Rha, Rhamnose; SCFA, short-chain fatty acids; TLR, Toll-like receptors; Xyl, Xylose;

Introduction

Relationship between the intake of plant-source foods and decrease in colorectal cancer risk

Cancer is one of the leading cause of death globally. Around one-third of cancer-related death are mostly connected to behavioural and dietary habits including tobacco and alcohol use, lack of physical activity, high body mass index, and low intake of fruits and vegetables [1]. Colorectal cancer (CRC) is known to be associated mainly with dietary patterns of the so-called western lifestyle. The incidence of CRC is higher in developing countries and this incidence is increasing fast in both low- and middle-income countries. This is mainly due to a shift in dietary patterns towards a decreased intake of plant-source foods and an increased intake of fat, sugar and animal-source foods [2]. Despite CRC is the second most diagnosed type of cancer in men and the third in women, as well as the third leading cause of all cancer death, only less than 10% of CRC arise from inherited syndromes [3,4]. Thus, studies have systematically pointed out that tackling modifiable risk factors, specially changing the dietary patterns, can substantially reduce CRC-related deaths [5–10].

Recently, a prospective longitudinal study revealed that a dietary pattern characterized by the high intake of plant-source foods is associated to a delayed CRC risk up to 10 years [11]. Systematic reviews and meta-analysis of case-control and cohort studies also reported an inverse association between the intake of plant-source foods and CRC risk [12–14]. Besides scientific data, the traditional knowledge suggests the intake of plant-source foods as adjuvant treatment against CRC [15]. As plant-source foods contain relatively high amounts of biologically active molecules, such as polyphenols and non-digestible carbohydrates (NDC), the adoption of specific nutritional interventions using fruits and vegetables has been taken into consideration to assist cancer therapies [16]. Furthermore, the increased ingestion of dietary fibre from plant-source foods, which are composed mainly by the NDC that constitutes the plant cell wall, is known for a long time to play a pivotal role in the reduction of CRC risk [17–20].

Although NDC are resistant to digestion by human enzymes, these carbohydrates are not a static collection of food components that pass through the gastrointestinal tract without inducing biological effects. Instead, NDC modulate nutrient absorption through binding to organic molecules that induce indirect biological effects acting as substrate for colonic fermentation by the gut microbiota [21]. Furthermore, recent efforts have focused on exploring the direct interaction between NDC and CRC cells that will be described further in this review. However, the composition and chemical structure of NDC may vary depending of plant species and tissues, thereby resulting in great heterogeneity of structure with variability in composition and branching pattern. Thus, although NDC from plant-source foods share common patterns and biological functions, the ingestion of these food components that have great variation in size and structure will result in structure-dependent properties and therefore diverse biological

effects. In this review, we will describe some known mechanisms through which NDC from plant-source foods induce beneficial health effects that relate to a decrease in CRC risk.

Structure of non-digestible carbohydrates from plant-source foods

As the chemical structure strongly influences the physicochemical properties and the biological effects of NDC from plant-source foods, it is necessary to define the main structural patterns of biologically active NDC in CRC models. NDC are comprised mainly by polysaccharides from plant cell wall, such as cellulose, hemicellulose and pectin (**Figure 1)**. Cellulose consists of relatively conserved polysaccharides with long and linear β-(*1,4*)-linked glucose (Glc) residues that vary in size according to plant species and tissue. On the other hand, hemicellulose consists of structurally complex and heterogeneous oligo- and polysaccharides with β-(*1,4*)-linked backbone of xylose (Xyl), Glc, mannose (Man) or galactose (Gal). The hemicellulosic fractions include xylans (glucoronoxylan, arabinoglucoronoxylan, arabinoxylan and other heteroxylans), mannans (acetylated and non-acetylated mannan, galactoglucomannan, galactomannan and glucomannan), galactans (galactan and arabinogalactan), xyloglucans, and mixed-linkage glucans [22], which vary in size and branching pattern.

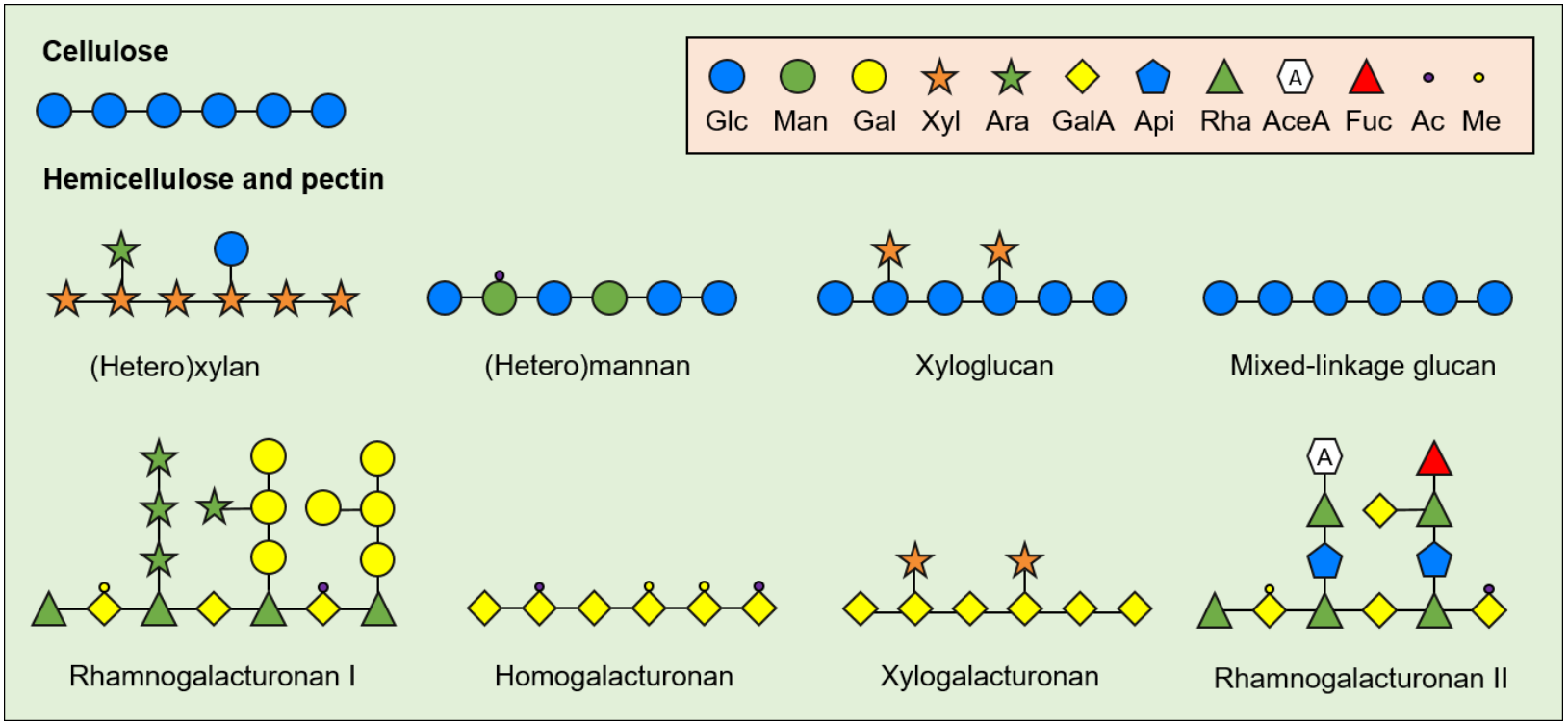

Figure 1. General structure of plant cell wall-derived non-digestible carbohydrates (NDC). Cell-wall derived NDC from plant-source foods include cellulose, hemicelluloses and pectin. Glc, Glucose; Man, Mannose; Gal, Galactose; Xyl, Xylose; Ara, Arabinose; GalA, galacturonic acid; Api, Apiose; Rha, Rhamnose; AceA, Aceric acid; Fuc, Fucose; Ac, Acetylated; Me, Methylated.

Similar to hemicellulose, pectin consists of linear and ramified homo- and heteropolysaccharides; however, pectin contains relatively high amount of acidic monomers compared to hemicellulose including mainly galacturonic acid (GalA). The major fraction of pectin usually consists of linear α-(*1,4*)-linked GalA residues (homogalacturonan, HG) with varying degree of methyl and acetyl esterification. Xylogalacturonan is also a component of pectin and have the same α-(*1,4*)-linked GalA backbone as HG but substituted at C2 and C3 with β-(*1,3*)-linked Xyl residues [23]. The pectic fraction also consists of branched structures named rhamnogalacturonan (RG-) I and II. RG-I is usually pointed out as the second major pectic fraction in plant-source foods and consists of a backbone of alternate α-(*1,4*)-GalA and α-(*1,2*)-rhamnose (Rha), in which the latter can be substituted at O4 mainly by arabinans, galactans and arabinogalactans – although substitutions with Xyl and Glc residues coexist in specific plant-source foods [24]. Finally, RG-II consists of an α-(*1,4*)-linked GalA backbone with complex branches made up of rare monomers (e.g. aceric acid and apiose) with different side chain, size and conformation depending on plant-food source [25–27].

As mentioned above, even though NDC is generally considered as a dietary fibre, the diversity of NDC structure from plant-source foods result in different physicochemical properties, fermentation patterns, and biological effects, thereby making the evaluation of the structure-function relationship challenging. Thus, there is an increasing number of studies exploring which specific structural patterns of NDC induce beneficial biological effects in CRC models [28–30].

Effects of the non-digestible polysaccharides on CRC development and progression

Studies have shown the association between the intake of specific food components and cancer, such as an inverse correlation between the intake of NDC from plant-source foods and CRC development and progression [12,18,31–33]. However, despite the evidence that high intake of NDC could reduce the risk of CRC up to 38% [34], the levels of this evidence is still considered as probable, because of both the broad spectrum of CRC subtypes [35], and the heterogeneity of physical and biological functions of NDC from distinct plant sources [36–38]. Besides that, there are the presence of others dietary components in food matrix that influence the physicochemical properties and biological effects of NDC [39]. Therefore, as some studies did not consider dietary components other than NDC in plant-source foods, such as polyphenols, vitamins, and minerals [34], it is difficult to establish an inverse association between the intake of NDC and CRC risk. A reliable characterization of the complex NDC structure, followed by their isolation, purification and the study of their biological effect, is crucial to reach a desirable structure-function relationship between NDC and the anticancer effects.

There are three main mechanisms in which NDC act against CRC development and progression. The consumption of NDC can induce (A) physicochemical effects in the gastrointestinal tract, (B) fermentation-related effects, and (C) direct effects resulting from the

interaction between NDC and cells, such as intestinal epithelial cells (IEC), immune system cells, and CRC cells (**Figure 2**). Below we summarized the physicochemical and the fermentation-related effects of NDC from plant-source foods, and focused on the recent findings that show the possible mechanisms through which distinct NDC directly interact with cells, thereby suggesting new beneficial effects regarding the intake of NDC and decreased CRC development and progression.

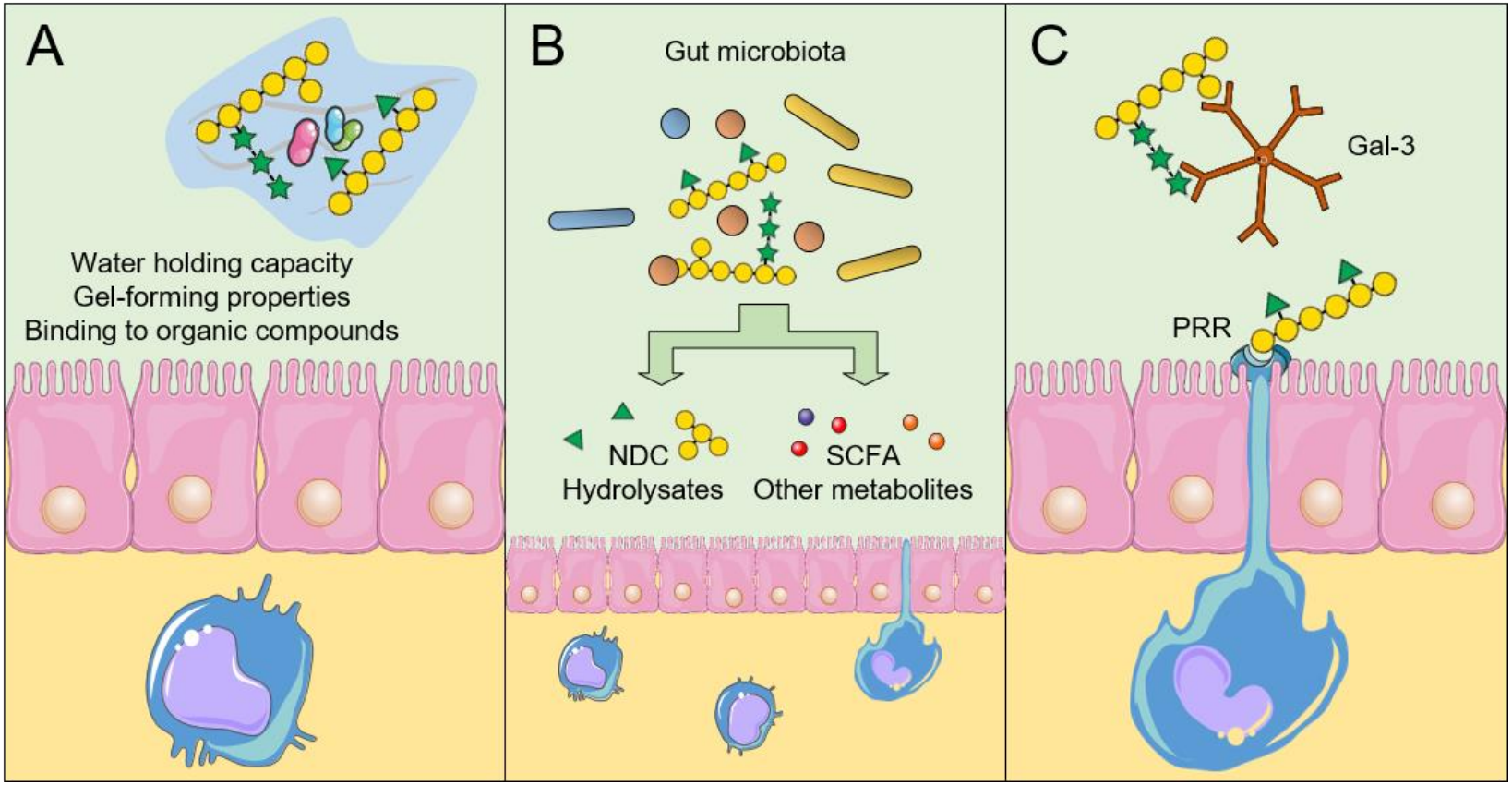

Figure 2. Physicochemical and biological effects of non-digestible carbohydrates (NDC) after the intake. (**A**) The physicochemical properties of NDC influence the absorption of other nutrients and reduce the interaction between carcinogens and the intestinal epithelium. Furthermore, NDC can promote an increase in satiety and stool bulk, as well as reduce the transit time throughout the gastrointestinal tract. (**B**) The fermentation-related effects result in the production of short-chain fatty acids (SCFA) and other metabolites, which can induce biological effects in epithelial intestinal cells, immune system cells and cancer cells. The fermentation of NDC by the gut microbiota can also influence the microbiota profile itself. (**C**) NDC can also interact directly with cellular components, such as the Pattern recognition receptors (PRR) and galectin-3 (Gal-3), thereby inducing downstream signalling pathways in cells and affecting cancer cell adhesion and invasion. The figure was modified from Smart Servier Medical Art (http,//smart.servier.com/), licensed under a Creative Common Attribution 3.0 Unported Licens (https,//creativecommons.org/licenses/by/3.0/).

Physicochemical effects

The physicochemical effects of NDC in the gastrointestinal tract are related to the interaction of these carbohydrates with other components through gel-forming properties, water holding capacity, and the ability of binding to other organic compounds [40].

Both gel-forming properties and water holding capacity result in increased stool bulk, thereby providing satiety [41]. Promising results concerning the effects of specific NDC on satiety have been stimulating industry to reformulate the nutritional composition of foods and community to change their dietary pattern, aiming to reduce obesity trends [42], which is a risk factor for CRC development [11,43]. The increase in stool bulk also contributes to the dilution of possible carcinogens. Furthermore, the reduction in stool transit time, which is a result of gastrointestinal mobility due to increased luminal content, reduces the exposition of IEC cells to carcinogens [44]. NDC can also entrap other food components or metabolites, thereby influencing macronutrient digestibility or metabolite reabsorption (e.g. glucose, lipids, bile acid) [37], and having a positive impact on postprandial insulin levels and glycaemic response. As example, β-glucan from barley, which consists mainly of linear and mixed β-(*1,4*)- and β-(*1,3*)-linked Glc polysaccharides, reduces postprandial glycaemic response improving glycaemic control [45]. Furthermore, β-glucan from distinct barley varieties bind to primary and secondary bile acids in intestine [46] and reduces bile acid reabsorption through the enterohepatic circulation, which is associated to a reduction of serum cholesterol levels [47,48].

The abovementioned physicochemical effects of NDC are dependent on both their macrostructure (e.g., molecular weight, degree of crystallinity, and particle size) and microstructure (e.g., presence of functional groups). In terms of macrostructure, studies suggest that β-glucans from cereals should have a molecular weight above 100 kDa to increase the viscosity of the digestive effluents and to induce a positive effect on postprandial response [49]. However, oat β-glucans with lower molecular weight have also increased bile acid capacity [50]. The overall structure is also a major source of variability in the physicochemical effects, since the threshold for arabinoxylans to induce a similar effect to that of β-glucans on postprandial response is significantly lower—approximately 20 kDa [49]. Furthermore, as the crystallinity of NDC influences their interaction with other components in the gastrointestinal lumen, changes in the crystallinity of NDC have a strong impact in their physicochemical effects. In this context, it was shown that rats fed with distinct celluloses with a degree of crystallinity ranging from 8 to 20% had differences in their faecal water content, which appears to be related to an inverse relationship between crystallinity and water holding capacity [51]. This inverse relationship is not observed only for cellulose [52], but also for other NDC from plant-source foods including galactomannans from coconut flour [53] and fenugreek [54]. The particle size of NDC also influences the physicochemical effects of NDC, as shown by the increase in water holding and lipid binding capacity of NDC from coconut after grinding [55]. However, other studies demonstrated that the reduction in the particle size of NDC decreased

the water holding capacity, as observed for NDC from rice bran [56], wheat bran [57], and citrus [58].

Recent studies that applied distinct processing methods (e.g., micronization, milling and enzymatic degradation) in NDC from plant-source foods also support the relationship between changes in both the degree of crystallinity and particle size with changes in the physicochemical effects [59–61]. For example, the reduction in the particle size of NDC from carrots subjected to high-pressure micronization, but not by ball milling, increases its water holding and lipid binding capacity [56].

In addition to the enzymatic- and physical-induced changes in the microstructure of NDC, studies are also exploring whether the introduction/removal of functional groups influences the physicochemical effects of specific NDC from plant-source foods. The phosphorylation of NDC from soybean does not appear to change its bile acid binding capacity. However, the water holding capacity of the phosphorylated NDC are 1.5-fold higher compared to the native NDC [62]. The degree of esterification also appears to be directly related to the water holding capacity of NDC, as was found for citrus pectin [63] and more recently for NDC extracted from eggplant [64].

Therefore, processing methods that affect the macrostructure or the microstructure of NDC can be applied to control the physicochemical effects of these dietary components. The knowledge and control in NDC characteristics may in turn be useful in for the selection and production of NDC from plant-source foods with desired physicochemical properties that are relates related to a decreased CRC risk.

Fermentation-related effects

The chemical structures of NDC are crucial for colonic fermentation because not all NDC are fermented, and because different metabolites resulting from the fermentation of distinct NDC act on a broad range of downstream signalling pathways in non-cancer cells and in CRC cells [65]. Besides the structure-dependent effects, the fermentation-related effects of NDC in the decrease of CRC risk are dependent of the gut microbiota itself since distinct bacteria profile will result in differentially bioactive metabolites production in a time- and structure-dependent manner [66–68].

Some bacteria from the human gut microbiota possess a large repertoire of enzymes that hydrolyse glycosidic linkages from complex carbohydrates to use the hydrolysates and some metabolites as energy sources [69]. However, the identification of key bacterial species in the gut microbiota that are responsible for the disassembling of specific NDC structural patterns remains somewhat limited [25,28,69–72]. Despite the questions that still need to be answered, the main outcomes of fermentation-related effects that contribute to a decreased CRC risk are the modulation of gut microbiota profile and the production of biologically active

metabolites including short-chain fatty acids (SCFA), such as acetate, propionate and butyrate [73]. The association between SCFA and the reduction of CRC risk was reviewed elsewhere [21,74].

SCFA produced after fermentation of NDC could help to maintain the lumen pH at lower levels, thereby inhibiting pathogens growth and favouring the establishment of a healthy gut microbiota. SCFA, especially butyrate, also stimulate IEC growth by functioning as the primary source of energy for these cells while being metabolized by β-oxidation in the mitochondria. Several mechanisms for SCFA uptake across the apical membrane of IEC had been proposed including transport by monocarboxylate transporter (e.g., MCT1 and SMCT1), counter-transport with bicarbonate, and passive diffusion [75]. These SCFA also act in downstream signalling pathways in CRC cells [76,77] and in non-cancer cells including IEC and immune system cells [78,79] through interaction with G protein coupled receptors (FFAR2/GPR43, FFAR3/GPR41, GPR109a and Olfr78) [80]. Thus, the uptake of SCFA by IEC results not only in the provision of energy to normal metabolic functions but also in the production of interleukin (IL-) 18 [81], involved in the maintenance of epithelial integrity, as well as in the increased secretion of antimicrobial peptides [82]. For example, butyrate reduces pro-inflammatory effects by inhibiting nuclear factor-κB (NF-κB) activation [83], as well as the Wnt signalling pathway, a pro-inflammatory pathway [84] constitutively expressed in some CRC cells [85]. Besides effects in CRC cells, butyrate contributes to the normal turnover of cells in the gastrointestinal tract, as it induces proliferation of IEC at the crypt of the colon and increases apoptosis of IEC at the villus [86]. Notably, this effect on proliferation does not occur at the same extent in CRC cells because cancer cells present a shift from oxidative metabolism to anaerobic glycolysis (the so-called Warburg effect), which results in the accumulation of lactic acid. As CRC cells rely on glucose as their primary energy source instead of butyrate, this shift in the metabolism of CRC cells results in accumulation of butyrate, whose increased intracellular levels inhibits histone deacetylases (HDAC), thereby resulting in cell cycle arrest and further induction of apoptosis in cancer cells [87] (**Figure 3**).

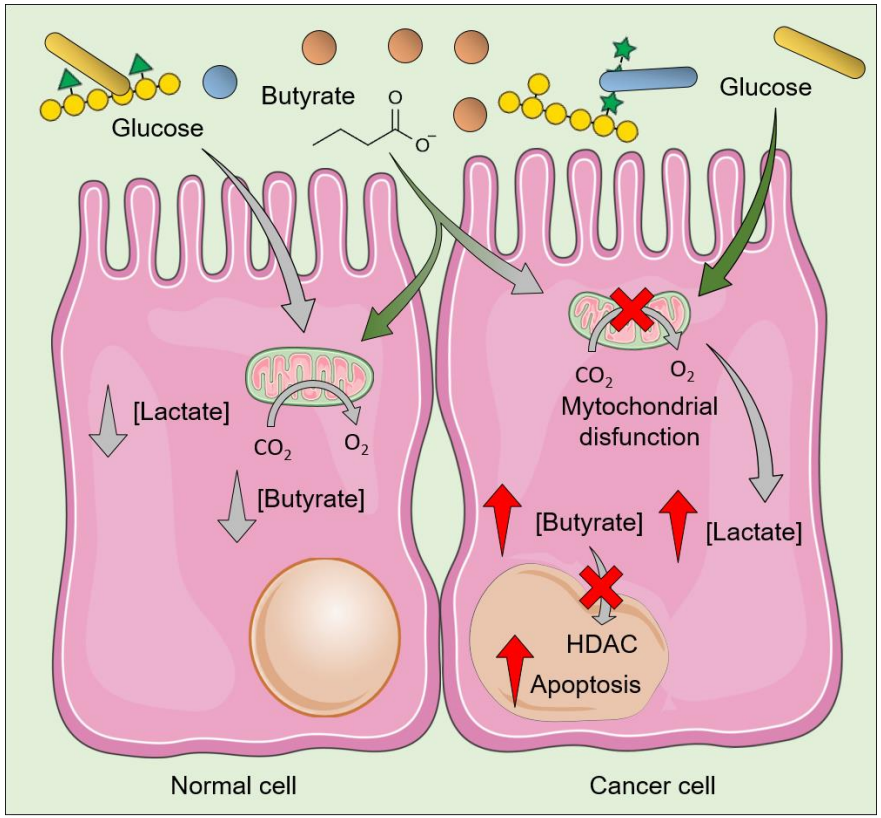

Figure 3. Effects of butyrate in normal cells and colorectal cancer cells (CRC). The butyrate produced during fermentation of non-digestible polysaccharides induces distinct effects in normal cell and CRC cells, as the latter rely on glucose—instead of butyrate—as their primary energy source. Increased glycolysis results in increased intracellular levels of lactate and decreased clearance/utilisation of butyrate, whose increased intracellular levels inhibit histone deacetylases (HDAC) and induce death of CRC cells. As normal cells usually butyrate as the main energy source, relatively low levels of butyrate is accumulated. The figure was modified from Smart Servier Medical Art (http,//smart.servier.com/), licensed under a Creative Common Attribution 3.0 Unported Licens (https,//creativecommons.org/licenses/by/3.0/).

In addition to the induction of IL-18 by IEC, which is also crucial for intestinal immune homeostasis since IL-18 helps maintaining the balance between T helper 17 cells (T_h17) and regulatory T cells (T_{reg}) [88,89], SCFA also interact directly with innate mechanisms of defence. In neutrophils, SCFA modulates recruitment, effector function, and cell survival [90]. Phagocytes including dendritic cells and macrophages also respond to SCFA, which regulates pro-inflammatory cytokine production [91,92].

Thus, despite CRC cells can use SCFA as energy source for proliferation [93], the fermentation-related effects on IEC and immune system cells have a clear relationship with the maintenance of host defence mechanisms and therefore with regulation of inflammatory response. As the molecular pathobiology of CRC usually implicates in pro-inflammatory conditions with an increase in the secretion of cytokines and chemokines that will promote malignant progression, invasion, and metastasis [94], the fermentation-related effects are generally regarded as an essential mechanism through which the intake of NDC relate with reduced inflammation and reduced CRC development and progression. For more detailed reviews regarding these fermentation-related effects of NDC in non-cancer and CRC cells, as well as the interplay between gut microbiota and fermentation-related beneficial effects, the reader is referred to Lam et al. [95], McNabney and Henagan [96], van der Beek et al. [97], and Zhou et al. [98].

Despite the mechanisms through which the fermentation-induced SCFA production relates to a decrease in CRC risk appear to be well known, recent studies have also been conducted to explore how specific NDC affect gut microbiota composition. As bacterial strains have distinct prebiotic properties, changes in the microbiota composition induced by these dietary components may influence SCFA production, thereby influencing CRC risk. A previous study strongly supports this hypothesis by showing changes in the microbiota composition of children from Europe and rural Africa during the transition between breast milk feeding and the introduction of solid diet [99]. The study has shown that differences between the faecal microbiota composition of children from Europe and rural Africa occurred only after the introduction of a solid diet. Children from Europe have reduced consumption of NDC from plant-source foods (e.g., arabinoxylans) compared to children from rural Africa, and this difference appears to induce an enrichment of Bacteroidetes phylum—whose members have specific genes encoding xylanases—in the gut microbiota of children from rural Africa. After this finding, numerous studies have applied *in vivo* analysis of faecal microbiota and/or *in vitro* human faecal fermentation to explore whether specific NDC induce changes in the microbiota composition, providing insights into the relationship between structure of NDC and their prebiotic function, as showed by the structure-dependent effects of NDC in promote the survival of *Lactobacillus* spp [100]. Furthermore, a recent study [101] explored the relationship between the structure of distinct NDC from orange, lemon, lime and sugar beet, and their beneficial effects on the modulation of gut microbiota. Using an *in vitro* colonic fermentation model (TIM-2), the authors had found that the increase in the degree of esterification of HG appears to be the most important parameter in determining beneficial effects on gut microbiota

composition, followed by the composition of neutral sugars (e.g., increase in HG/RG ratio and the presence of arabinose) and the reduction in the degree of branching [101].

Thus, although studies are successfully proving insights into the relationship between the structure and prebiotic function of purified NDC from plant-source foods, the preference of a specific bacterial strain in utilize an NDC from a food matrix appears to be more complex, as the fermentation rate of a single NDC is affected when others NDC and other dietary components (e.g., polyphenols) are present [39]. In this context, it was found that mixing fast-fermenting NDC including HG from citrus pectin and xyloglucan from tamarind reduces their fermentation rate, thereby delaying the prebiotic effect [102]. This reduction in the fermentation rate probably make NDC reach the distal parts of the colon, which is of particular importance in terms of CRC risk. The reduced fermentation rate in the distal part of the colon have been thought as one of the reasons why most of CRC are detected in this region. This delay in the prebiotic effect by mixing different NDC appear to occur because of the hierarchical preference, which refers to the ability of a bacterial strain in prioritize the utilisation of some NDC before others [103]. Studies confirm the hierarchical preference by observing that a bacterial strain can prioritize the fermentation of specific host mucosal glycans [104] or NDC from plant-source foods [105]. Examples of hierarchical preference includes the preference of *Bacteroides thetaiotaomicron* in utilising galactan from potato instead of arabinan from sugar beet [103], as well as the increased ability of *Bacteroides* spp., *Bifdobacterium* spp., *Faecalibacterium* spp., and *Lactobacillus* spp. in utilise fructans with low molecular weight compared to fructans with high molecular weight [106,107]. These hierarchical preferences appear to be strain-specific, as closely related gut bacterial strains (e.g., *B. thetaiotaomicron* and *B. ovatus*, or *L. delbruckii* and *L. paracasei*) prioritize the use of distinct NDC [71,108]. Furthermore, recent studies are focusing on evaluate whether distinct gut microbiota profiles can utilize specific NDC from plant-source foods, such as arabinoxylans from corn and sorghum, and fructans from chicory root [105,109]. Therefore, studies using more complex samples such as the whole food or a mix of NDC instead of using a single NDC, as well as studies comparing the ability of distinct microbiota profiles in utilizing the same NDC—as performed by Yang et al. [110], Chen et al. [105] and Brahma et al. [111]—, are elucidating practical knowledge required to use prebiotic therapy or diet modifications to benefit the function of specific bacterial strains that relates to a decreased CRC risk.

Direct effects

NDC share structural features to lipopolysaccharides and other structural carbohydrate-containing molecules at the surface of bacteria [112,113]. As these carbohydrates from bacteria directly interact with IEC and immune system cells along the gastrointestinal tract, it was hypothesized that NDC from plant-source foods also directly interact with cells in the gut.

The abovementioned hypothesis has been confirmed through *in vitro* studies and most recently through *in vivo* studies [114]. Since pattern-recognition receptors (PRR) in cells are

the main responsible for the recognition of bacterial carbohydrates, efforts have been made mainly on the investigation of the PRR-mediated effects of NDC [115], although some direct but PRR-independent mechanisms have also been described [116,117] and will be pointed out later in this review.

Pattern recognition receptor-mediated effects

PRR existing in IEC and immune system cells regulates epithelial proliferation and intestinal permeability, and maintains gut homeostasis through recognition of harmful organisms and endogenous metabolites [118]. Furthermore, PRR plays an important role in shaping intestinal microbiota in both composition and number by interacting with commensal bacteria [119]. Thus, PRR-mediated signalling pathways result in immune surveillance and in maintenance of host-bacteria interaction alongside the gastrointestinal tract, whose dysregulation is clearly associated with increased CRC risk [98,120]. As NDC influence microbiota profile and therefore the formation of specific metabolites in the gut lumen, the intake of plant-source foods rich in NDC influences the PRR-mediated responses through an indirect mechanism. Interestingly, NDC also directly interacts with PRR in a structure-dependent manner [114,115].

The PRR include RNA helicases (RLR), Nucleotide binding oligomerization domain (NOD)-like receptors (NLR), C-type lectin receptors (CLR) and Toll-like receptors (TLR), which recognize distinct evolutionarily conserved pathogen-associated molecular patterns (PAMP) of microorganisms—such as the carbohydrate-containing molecules at the surface of microorganisms—as well as endogenous damaged-associated molecular patterns (DAMP) [120]. Despite the variety of PRR in human cells, only few PRR has been shown to recognize NDC from plant-source foods (**Table 1**). Most of studies focused in the interaction between NDP from plant-source foods and TLR, but we will also describe some recent findings regarding the effects of NDC from foods through NLR- and CLR-dependent pathways.

Table 1. Pattern-recognition receptors (PRR) that recognizes non-digestible carbohydrates (NDC) from plant-source foods. NLR, Nod-like receptors; CLR, C-type lectin receptors; TLR, Tool-like receptors; HG, Homogalacturonan; RG-II, Rhamnogalacturonan II; AG, Type II arabinogalactan; ME, Methyl esterification; RS, Resistant starch; FOS, Fructooligosaccharides.

PRR	NDC	Plant-source food	Effects	Ref
NLR				
NOD1-2	HG, RG-II and AG-II	Papaya (ripe)	Activation in HEK cells, NF-κB release	[121]
NOD2	Inulin	Chicory root	Activation in HEK cells, NF-κB release	[115]
NLRP3	HG, RG-II and HC	Chayote	Inhibition of NLRP3 priming in macrophage-like cells	[122]
CLR				
Dectin-1	Mixed linkage β-glucan	Barley	Activation in immune cells, NF-κB release, IL-6 and IL-8 release	[123]
Dectin-1	Arabinoxylan	Wheat	Inhibition in HEK cells	[124]
TLR				
TLR2	Inulin	Chicory	Activation in THP-1 cells, NF-κB release	[115]
TLR2	RS2	Maize	Activation in HEK cells, NF-κB release	[125]
TLR2	Maltooligosaccharides	Wheatgrass	Activation in immune cells	[126]
TLR2 and 4	FOS	Rice	Induction of dendritic cell maturation in mice	[127]
TLR2 and 4	HG (varying degree of ME)	Lemon	Activation in T84 cells, maintenance of intestinal epithelial barrier integrity	[128]
TLR2 and 5	RS3	Maize	Activation in HEK cells, NF-κB release	[125]
TLR2, 3, 5, 9	HG, RG-II, AG-II and HC	Papaya (ripe)	Activation in HEK cells, NF-κB release	[121]
TLR2, 3, 9	HG, RG-II, AG-II and HC	Papaya (unripe)	Activation (TLR2) or inhibition (TLR3 and TLR9) in HEK cells	[121]
TLR4	Galactan	Apple	Inhibition of LPS-induced activation in a colitis model	[129]
TLR4	HG (varying degree of ME)	Citrus	Inhibition of LPS-induced activation in a colitis model	[130,131]
TLR4	HG (branched)	Citrus	Inhibition in immune cells	[130]

Continue...

PRR	NDC	Plant-source food	Effects	Ref
TLR4	Levan	Soybean	Cytokine release in mice	[132]
TLR4-8	Inulin	Chicory	Activation in THP-1 cells, NF-κB release	[115]
Heterodimers				
TLR1\TLR2	HG (low degree of ME)	Lemon	Inhibition of intestinal inflammation	[133]
Dectin-1\TLR2	Galactomannan	Guar gum	Inhibition of IEC *in vitro* and in a colitis model	[134]
Dectin-1\TLR2	Galactomannan	Guar gum	Inhibition of IEC in a colitis model	[135]

Nucleotide binding oligomerization domain-like receptor

All NLR have C-terminal leucine-rich repeat motifs (LRR) for ligand sensing, except NLRP10 [136], and a NACHT domain that facilitates protein oligomerization [137]. The presence of the LRR motif and NACHT domain are essential for NLR function, which acts as a scaffolding cytosolic protein that assembles signalling platforms triggering NF-κB and mitogen-activated protein kinase (MAPK) signalling pathways, thereby controlling the activation of several caspases [137]. Thus, the activation of NLR usually results in the assembling of pro-inflammatory complexes termed inflammasomes, which relates not only to NF-κB- and MAPK-mediated secretion of cytokines and chemokines but also to the activation of a myriad of cell death regulators [138]. Therefore, the dysregulation of NLR have been associated with infections, autoimmune diseases, inflammatory disorders and cancer, such as CRC [139,140].

Among the more than 20 NLR that have been identified in human cells [141], NOD1, NOD2 and NLRP3 are pointed out as the most important in terms of relevant biological functions and CRC development [138]. The mechanisms through NLR-mediated effects are associated with increased CRC risk relates mainly to an excessive NLR-induced chronic pro-inflammatory microenvironment [142]. Intriguingly, NLR agonists, specially NOD1 and NOD2 agonists, have been proposed as therapeutic agents in CRC treatment because both experimental studies showed that NOD1 deficiency leads to increased tumorigenesis in mice [143] and the activation of these NLR may regulate the pro-inflammatory effects induced by other PRR [144,145]. Thus, although both beneficial and deleterious effects of NLR activation on CRC remains not fully understood, it seems that punctual activation or inhibition of NLR could differentially induce effects during cancer development, progression, and metastasis.

Despite the relevance of NLR in CRC risk, few studies had focused on exploring the effects of NDC from plant-source foods in the regulation of NLR because they are cytosolic receptors. Phagocytes including macrophages can internalize NDC [146], but the exact mechanism of interaction between NDC and cytosolic receptors is not proven. Furthermore, it is known that IEC can transfer exosome-like vesicles from their apical or basolateral surface to both mesenteric lymph node and gut lumen [147], and these cells can also take up exosomes from foods (e.g., bovine milk exosomes) or produced by immune system cells [148]. However, despite these evidences, it is not possible to assert that vesicle trafficking between cells—or between cells and food components—is responsible for the internalization of NDC. Despite the lack of evidences on the mechanisms through which NDC interact with cytosolic receptors, our research group had recently found that NDC from ripe papaya, but not from unripe papaya, activated both NOD1 and NOD2 in an *in vitro* model using HEK 293 hNOD1 and HEK 293 hNOD2 reporter cells. The distinct effects of NDC from ripe and unripe papaya on NLR activation seem to be related to changes in the structure of NDC that occur during fruit ripening, as ripe papaya had increased proportion of homogalacturonans with higher degree of methylation compared to NDC from unripe papaya [121]. Notably, a similar NDC—also from papaya fruit—inhibited CRC cells proliferation *in vitro* [149]. Furthermore, linear β-(*1*,*2*)-linked fructose

oligosaccharides (inulin) from chicory root also activate NOD2 in HEK 293 hNOD2 reporter cells [115].

On the other hand, NDC from chayote fruit, which consists mainly of pectic homogalacturonan and highly branched RG-II, as well as hemicellulosic material including glucomannan, xyloglucan, and glucurono(arabino)xylan, inhibits NLRP3 inflammasome activation in human THP-1 macrophage-like cells. The effects of this NDC on NLRP3 inflammasome can be considered an indirect effect of the interaction between the NDC and other PRR that are essential to induce priming signals required for NLRP3 inflammasome activation [122]. In fact, cross-talk between PRR may occur through three main mechanisms. It can be a (a) requirement of two or more PRR for a specific biological response, (b) an interaction between PRR for robustness or redundancy of biological response, or (c) a negative regulation between PRR [150]. Thus, although the effects of NDC from chayote were not directly explored in CRC cells, it can be proposed that NDC-mediated effects on the modulation of cytosolic NLR may occur through indirect mechanisms that involve the interaction between NDP and other PRR. Therefore, despite the investigation of NDC-induced effects on NLR is important to gain further insights into the biological effects induced by NDC from plant source foods, further analyses are necessary to explore the interaction between the NDC and other PRR, such as CLR and TLR.

C-type lectin receptors

Unlike NLR that are cytosolic proteins, the main CLR (Dectin-1, Dectin-2, Mannose receptor, and macrophage inducible Ca^{2+}-dependent lectin—Mincle) are trans-membrane PRR widely expressed in myeloid cells. Glycosylated structures are the natural ligands of CLR, which contain conserved carbohydrate-recognition domains (CRD). Thus, it is easy to think that some food-derived carbohydrates interact with CLR. In this regard, studies had explored the interaction between NDC from foods and CLR, especially Dectin-1. However, the effects were investigated mainly using fungal-source foods [151–153] compared to plant-source foods [123,134]. As CLR is expressed mainly in antigen-presenting cells including macrophages and dendritic cells, the study of the interaction between NDC from foods and CLR focused mainly on innate immune responses against pathogens and cancer, including CRC.

Activation of CLR can induce anti-inflammatory effects, as observed by the activation of the heterodimer Dectin-1\TLR2, which increases suppressor of cytokine signalling (SOCS)-1 expression, thereby resulting in anti-inflammatory effects [154]. However, in general, the inhibition of CLR in some CRC cells [155] promotes cell apoptosis [156] and suppresses a pro-inflammatory phenotype, thereby reducing CRC risk [157]. As CLR also facilitates adhesion of head, neck and breast cancer cells to the lymphatic endothelium and thus favour tumour invasiveness [158], NDC-mediated inhibition of CLR may directly impact in the invasiveness of cancer cells. On the other hand, CLR is essential for the recognition of altered glycosylated membrane proteins of CRC cells by immune system cells [159,160]. Thus, as NDC-mediated inhibition is

beneficial in cancer cells but may suppress anticancer response by the innate immune system, further studies using *in vivo* models may elucidate possible benefits on the interaction between NDC from plant-source foods and CLR in decreasing CRC risk.

Among all CLR, Dectin-1 seems to have a major impact in innate immune responses against cancer and are present in CRC cells [155,159]. Dectin-1 is a specific receptor for β-glucan [161,162], which is the most abundant fungal cell wall polysaccharide – and is also a constituent of the bacterial cell wall [163,164]. As β-glucan is a naturally occurring NDC in mushrooms and some plant-source foods, especially in cereal grains [165], Dectin-1 is by far the most studied CLR in terms of interaction with food-derived NDC. Furthermore, as Dectin-1 expression is high in phagocytes such as macrophages and dendritic cells, β-glucan from foods seems to act first through interaction with these innate immune system cells [166].

Phagocytes can extrude their dendrites across the intestinal epithelium into the gastrointestinal lumen and diet-derived β-glucan could interact with them through Dectin-1. Upon activation of Dectin-1, β-glucan induces mainly the Spleen tyrosine kinase (Syk)-dependent pathway, which triggers adaptive immune response in T cells and B cells that results in the inhibition of both tumour growth and metastasis [167]. Despite the inhibition of Dectin-1 induces apoptosis in CRC cells *in vitro* [156], evidence that showed the positive role of Dectin-1 activation in the decrease of CRC risk comes from the findings that a loss of function of this CLR is associated with increased risk of ulcerative colitis [168]. Furthermore, the relationship between the loss of Dectin-1 function due to polymorphism and the increasing risk of inflammatory disorders in the gastrointestinal tract [160], as well as other *in vivo* studies [169,170], supports the beneficial effects of Dectin-1 activation in the reduction of CRC risk.

In this context, a barley-derived β-glucan that consists of linear and mixed β-(*1,4*)- and β-(*1,3*)-linked Glc residues interacts with Dectin-1 and triggers a Syk-dependent pathway that results in the activation of NF-κB of immune system cells, leading to cytokine secretion including IL-6 and IL-8 [123]. Thus, it is possible that some NDC from plant-source foods, especially β-glucans, directly activates Dectin-1 and induces positive effects in the reduction of CRC risk. In addition to enhancing the innate immune response, CRL seems to function together with other PRR, especially with TLR [153,171,172], to regulate the function of IEC. In this context, a recent study showed that guar gum exerted *in vitro* and *in vivo* anti-inflammatory effects in IEC through a Dectin-1\TLR2-dependent pathway [134]. The same mechanism seemed to be related to the anti-inflammatory effects of partially hydrolysed guar gum—which consists of a backbone containing β-(*1,4*)-linked Man residues and short branches containing Gal at C4—in a colitis model [135].

Toll-like receptors (TLR)

TLR are the most studied class of PRR because of both the variety of PAMP and DAMP that interact with these germline-encoded PRR and also because of the biological outcomes that TLR-induction/inhibition could cause in human health [173]. Currently, 13

TLR have been identified in human cells, among intracellular and extracellular receptors [174]. As NLR, TLR are evolutionary conserved PRR-containing LRR motifs for ligand sensing that induces NF-κB and MAPK signalling. Most of TLR activate NF-κB and MAPK mainly through the adaptor myeloid differentiation factor 88 (MyD88), except TLR3, which triggers NF-κB and MAPK through a TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway [175].

Among all TLR, TLR2 and TLR4 have been the most studied ones concerning the interaction with NDC from plant-food sources [115,128]. TLR2 recognizes different kinds of PAMP including lipoprotein, lipoteichoic acid and peptidoglycan molecules from Gram-positive bacteria, as well as DAMP, such as heat shock proteins (HSP). TLR4 interacts with lipopolysaccharide (LPS) from Gram-negative bacteria and with several DAMP including HSP, fibronectin and heparan sulfate [176].

In the context of CRC, several studies had explored the role of TLR on cancer development, progression, and invasion, as reviewed by Li et al. [177]. Furthermore, results of a recent prospective cohort study suggested that the protective effect of NDC on CRC risk may involve interactions between the NDC and TLR4, and that polymorphisms in TLR2 and TLR4 are associated with increased CRC risk [178]. In fact, CRC development and progression have been correlated with TLR2 and TLR4 overexpression in CRC [179]. CRC cells express both mutated TLR2 and MyD88, thereby resulting in increased activation of TLR2-dependent pathways. Thus, TLR2 inhibitors were proposed as therapeutic agent in CRC [180].

TLR4 is considered the most important inflammatory inducer amongst all TLR, thereby playing a key role in immune response against intestinal pathogens. However, excessive activation of TLR4 may enhance not only immune response but also gives rise to cancer progression through disruption of intestinal immune homeostasis [181]. Excessive TLR4 activation also induces macrophages apoptosis after activation of the receptor-interacting serine/threonine-protein kinase (RIPK) 1 and RIPK3, which induces cell lysis and necroptotic death [182]. Furthermore, enhanced expression of TLR4 in CRC cells promotes cell survival, epithelial-mesenchymal transition [183], and downregulates the expression of the death receptor Fas in cancer cells [184,185]. Thus, excessive activation of TLR4 increases the risk of inflammatory diseases and CRC, and—as occur to TLR2—potential inhibitors of TLR4/NF-κB pathway have also been considered as therapeutic agents in CRC [186].

Despite the abovementioned evidences show that inhibition of TLR4-dependent signaling pathways may reduce CRC risk, some NDC from plant-source foods including citrus pectin and ginseng polysaccharides have potential anticancer effects that seems to be related to TLR4-mediated activation [128,187–189]. The most reasonable explanation is that these NDC from plant-source foods do not activate TLR4 at the same extent as natural PAMP, such as LPS. Therefore, NDC-induced TLR4 activation reduces the interaction of this PRR with more potent ligands [188–190]. Furthermore, TLR-mediated activation can

launch a strong immune response to assist cancer treatments and/or activate TLR-dependent programmed cell death, including apoptosis, autophagy and necroptosis [191].

Apart from the biological relevance of TLR2 and TLR4, TLR3 activation with polyinosinic, polycytidylic acid induces apoptosis of in CRC cells [192], whereas TLR5 activation suppress CRC growth and induce necrosis of cancer cells *in vivo* [193]. Furthermore, TLR9-induced activation in immune system cells promotes cell survival and therefore enhances immune response against cancer; however, the role of TLR9 in CRC remains unclear [177], as CRC cells have reduced expression of TLR9, suggesting a protective role of TLR9 expression against malignant transformation in the gastrointestinal tract [194].

Molecules from plant-source foods including polyphenols [195,196] and NDC have been found to exert effects on TLR-mediated pathways [114,115,133]. In the context of NDC from plant-source foods, it was found that apple galactan suppressed LPS-induced activation of TLR4 downstream signaling in an *in vivo* model of colitis-induced CRC [129]. Furthermore, NDC from apple reduced the migration of CRC cells *in vitro* [197], and enhanced the inhibitory effect of 5-fluorouracil in the growth of CRC cells [198]. Homogalacturonan-rich fractions from lemon pectin with varying degree of methyl esterification also induced TLR2- and TLR4-mediated responses [128]. Authors have showed that the varied degree of methyl esterification in the homogalacturonan residues of lemon pectin strongly influenced TLR2-mediated responses, but did not affect TLR4-mediated response. Furthermore, both low- and high-methoxylated lemon pectin seemed to exert positive effects on the maintenance of intestinal epithelial barrier integrity *in vitro* [128]. Recently, it was found that the inhibitory effect of low-methoxylated pectin from lemon suppressed the pro-inflammatory TLR2\TLR1 pathway while the heterodimerization between TLR2 and TLR6, which induces a tolerogenic effect, was not induced by lemon pectin [133]. Furthermore, authors found that the administration of low-methoxylated pectin from lemon prevented intestinal inflammation *in vivo* in a fermentation-independent manner. Notably, similar TLR-inhibitory effects are in agreement with *in vivo* studies using low- and high-methoxylated citrus pectin, which attenuated both endotoxin shock through a TLR-dependent pathway [130], as well as inflammatory effects in a colitis model [131].

Besides the TLR-mediated effects of NDC from apple and citrus, it was found that inulin from chicory roots with distinct chain-lengths interacted not only with TLR4, but also with TLR5, TLR6, TLR7 and TLR8 in a MyD88-dependent pathway, and had no effects on cytosolic TLR3 and TLR9 [115]. Furthermore, it was found that water-soluble NDC from unripe and ripe papaya differentially regulated TLR3, TLR5 and TLR9. Notably, NDC from ripe papaya activated TLR3, TLR5 and TLR9, whereas NDC from unripe papaya blocked TLR3 and TLR9, and had no effect on TLR5 [121]. Although it is not clear the biological outcome related to the interaction between these NDC and TLR, the observation of TLR-mediated effects by NDC from plant-source foods may support further studies aiming to evaluate the direct effects of these dietary components in CRC development and progression.

Direct interaction with galectin-3

In addition to the direct effects of NDC from plant-food sources through PRR-dependent mechanisms in IEC, immune system cells and CRC cells, NDC can also directly interact with cellular components in a PRR-independent pathway. The main PRR-independent effect seems to be related with the interaction between NDC from plant-source foods and the galectin-3 (Gal-3).

Gal-3 is a protein of the lectin family that has a CRD with strong affinity for β-galactosides. Notably, it has been consistently shown in the past two decades a strong association between increased levels of Gal-3 and several types of cancer including CRC [199,200]. Gal-3 expression had been correlated not only with CRC incidence, but also with CRC severity, as increased levels of Gal-3 are associated with a worse cancer prognostic [199–201]. A recent study supported this correlation by showing that the positive expression rate of Gal-3 in CRC tissues is approximately 5-fold higher compared to cancer-adjacent tissues [200]. Furthermore, authors suggest a direct association between positive expression of Gal-3 and both tumour size and malignant progression.

Gal-3 is present intracellularly—at the cytoplasm or within the nucleus—attached to cell surface, or in the extracellular media as a dimer or as a pentamer [202]. Regardless the localization of Gal-3, the increase in its levels is related with increased CRC risk and severity because this protein is involved in a wide range of cancer-promoting effects including CRC cells adhesion, invasiveness, growth and proliferation [203,204]. Notably, some NDC from plant-source foods bind to the CRD of Gal-3 and therefore inhibits Gal-3-mediated effects, which includes not only attachment to glycan-containing surfaces [205], but also with downstream signalling mechanisms that inhibits both apoptosis and cell cycle arrest in CRC cells [206]. Furthermore, Gal-3 appears to be associated to multiples mechanisms related to chemo-resistance of CRC cells by enhancing drug efflux, DNA repair mechanisms and activating signalling pathways (e.g., Wnt, Hedgehog and Notch) associated with multi-drug resistance [207]. Thus, since the observation of specific bind of NDC from plant-source foods to Gal-3 [208], several studies have been performed to assess the interaction between distinct NDC and Gal-3, as well as the effects of this interaction in CRC progression, as described previously [117].

Among NDC from plant-food sources and Gal-3 inhibition, the modified citrus pectin (MCP) is the most studied one [187,209–211]. MCP is a preparation derived from citrus pectin that is modified by high temperature, alteration of pH and/or pectinase treatment, which result in the partial hydrolysis of glycosidic linkages, thereby generating smaller and less ramified NDC structure. These processes of MCP modification release neutral chains of galactan with high affinity to the CRD of Gal-3 [208,209], which induce a broad range of inhibitory effects that had been extensively studied through *in vitro* and *in vivo* studies [209,212,213].

Modified sugar beet pectin, papaya pectin, and ginseng pectin have structures composed of neutral (*1,4*)-β-galactose residues, which were related to Gal-3 interaction and

inhibition [210,211,214]. As observed for MCP, the binding between these pectin and Gal-3 has been associated with both *in vitro* and *in vivo* effects on CRC [211,215]. Besides the interaction with Gal-3, molecular size-fractionated MCP showed other effects than inhibit Gal-3, as leading CRC cells to apoptosis and inhibiting their migration [216]. NDC from plant-source food also seem to interact with Gal-3 through non-specific binding as suggested by previous study [217]. Since homogalacturonans contain relatively high amounts of charged GalA residues, it is possible that charge-charge interactions between GalA residues and Gal-3 induce a non-specific binding that may exert inhibitory effects on Gal-3 activity. Recently, it was also shown that NDC from plant-source foods can interact with Gal-3 by a combination of homogalacturonan and RG residues acting in concert, as the homogalacturonan seem to interact with RG exposing additional galectin-binding sites of the NDC, thereby enhancing Gal-3-binding properties [218].

As NDC are essentially polyhydroxy molecules, which are often esterified, it is possible that the NDC from plant-source foods interacts with other cellular components. The studies that had shown anticancer effects of NDC through interaction with Gal-3 support further studies aiming the investigation of the interaction between NDC and other signalling mediators related to the decreased CRC risk.

Concluding remarks

The complexity of biological effects resulting from the intake of NDC from plant-source foods and their relationship with decreased CRC risk can be divided into physicochemical effects, fermentation-related effects, and PRR-dependent and PRR-independent direct effects. However, in biological systems, these complex NDC effects occur at the same time in an intricate – and poorly understood – relationship. Although the evaluation of a specific biological effect does not fully answer whether a single NDP from a plant-source food relates to a decreased CRC risk, it can provide further insights to elucidate the structure-function relationship between NDC and their effects in CRC development and progression. Therefore, as recent studies are demonstrating that intrinsic properties of NDC from plant-source foods, as well as individual characteristics among cells and individuals, strongly influence the beneficial effects of NDC on the reduction of CRC risk (**Figure 4**), similarities between the intrinsic properties of NDC from distinct plant-source foods may drive the discovery of new bioactive NDC. Clearly, studies that integrate the structural characterization of NDC from plant-source foods with their physicochemical, fermentation-related, and/or direct effects will provide insights not only for a better understating of the structure-function relationship between the intake of NDC and CRC risk but also for improving nutritional recommendations of NDC for healthy individuals, as well as CRC patients.

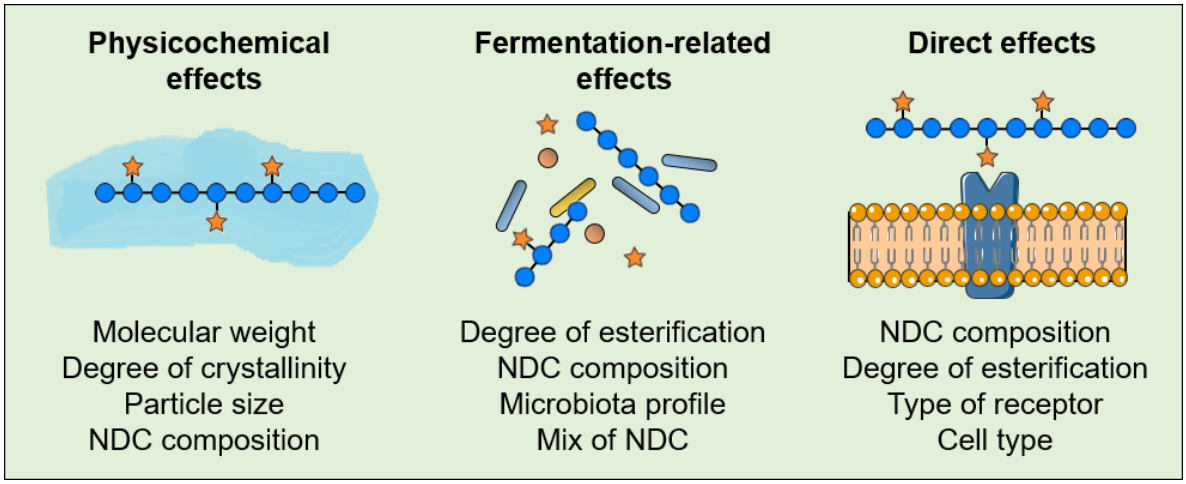

Figure 4. Features that influence the effects of non-digestible carbohydrates (NDC) from plant-source foods in colorectal cancer (CRC). Some of the intrinsic properties of NDC, as well as individual characteristics among cells and individuals, that influences the physicochemical, fermentation-related and direct effects of NDC from plant-source foods on the reduction of CRC risk.

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Chapter 4

Part I: Migration and proliferation of cancer cells in culture are differentially affected by molecular size of modified citrus pectin

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Chapter 4

Part II: Ripening-induced chemical modifications of papaya pectin inhibit cancer cell proliferation

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Chapter 5

Ripening processes in papaya creates unique pectin structures with differential signaling effects on pattern recognition receptors

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Abstract

Dietary fiber consumption is known to exert healthy benefits and more recently was associated to be able to interact with pattern recognition receptors (PRR). The interaction with these receptors, like toll-like receptors (TLR) and nucleotide binding oligomerization domain (NOD)-like receptors can modulate immune responses. The papayas pectin extracted from unripe and ripe fruits exerts structural-dependent effects on cancel cell lines, and the aim of this work was evaluating whether the unripe and ripe papayas pectin also has different interactions with PRR. The pectin from ripe papayas activate TLR and in a less extension NOD receptors. The pectins from unripe papayas also activated some TLR but not for TLR3 and TLR9. The pectin from unripe papayas not activated TLR3 and TLR9 and also block the agonist activation. The main difference in the pectin structure is higher esterification and smaller chains of pectin from ripe papayas. Therefore, the distinct biological effects using papayas pectin can be due to the different PRR interaction.

Keywords: pectin; papaya; pattern recognition pattern; toll-like receptors.

Abbreviations: AFM, atomic force microscopy; Ara, arabinose; ATR, attenuated total reflectance; DAH, days after harvest; DM, degree of methyl-esterification; FTIR, fourier transform infrared; Fuc, fucose; Gal, galactose; GalA, Galacturonic acid; Glc, glucose; GlcA, glucuronic acid; HG, homogalacturonan; I-WSF, intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; LPS, lipopolysaccharides; Man, mannose; Mw, molecular weight; NOD, nucleotide-binding oligomerization domain; PRR, pattern recognition receptors; R-1-WSF, ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF, ripe - papaya from 5th day after harvest - water-soluble fraction; RG-I, type I rhamnogalacturonans; Rha, rhamnose; SD, standard deviation; SEAP, soluble embryonic alkaline phosphatase; TLR, toll-like receptors; Un-1-WSF, unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF, unripe - papaya from 2nd day after harvest - water-soluble fraction; V_e, elution volume; V_o, void volume ; WSF, water-soluble fraction; Xyl, xylose; DF, dietary fiber.

Introduction

Dietary fiber (DF) commonly represents a wide variety of polysaccharides originating from fruits, vegetables, whole grains and legumes. Consumption of DF has been shown to influence the consumers health status including its immune response. This can be accomplished by direct effects of DF on consumer's immunity or by beneficial effects on gut microbiota that ferment DF into short chain fatty acids that may influence immune regulation[1]. DF also induces a slower, desired gastric emptying [2], improves physical bowel function [3], and can interact directly with intestinal cells and/or the cells from the mucosal immune [4–6].

The direct interaction of DF with the intestinal cells can be throughout Pattern Recognition Receptors (PRR) [7]. The PRR are germline-encoded sensors expressed in intestine epithelial cells and gut immune cells, being the key receptors responsible for the recognition of exogenous molecules by the host [6,7]. Toll-like receptors (TLR) is a family of PRR and play a central role in the activation of innate immunity [8] and have been shown to be involved in DF induced immune signaling. The immune response mediated by TLR activation requires the recruitment of MyD88 adaptor protein and the translocation of NF-κB to the nucleus [8], unless TLR3 that NF-κB is activated mediated TIR domain-containing adapter inducing IFN- β (TRIF) [9]. The interaction between a wide variety of DF-polysaccharides and TLR has been extensively studied and is extremely complex, some DF activates TLR to different extends [10] while other DF such as pectins may block TLR signaling and attenuate intestinal inflammation [11]. Nucleotide-binding oligomerization domain (NOD) have also been shown to be influenced by DF such as β2→1-fructans. NOD are proteins responsible for the recognition of intracellular bacteria [8]. Through this signaling via PRRs DF have been shown to mediate several host effects such as reducing intestinal permeability and thereby supporting gut barrier function [10,12], supporting immune responses against pathogens [13], and reducing intestinal inflammation [11].

The DF isolated from fleshy fruit is mainly formed by cell wall-derived polysaccharides: cellulose, hemicelluloses and pectin [14] with the most water-soluble fraction being composed of pectin. Papaya (*Carica papaya* L.) is a climacteric fruit which ripens fast resulting in fruit softening [15]. The ripening-induced expression of cell wall-degrading enzymes is responsible for cell wall disassembling with concomitant changes in papaya DF structures, e.g. through the generation of water-soluble pectins during ripening

[16]. The papaya water-soluble fraction (WSF) isolated from pulp is mainly composed of pectin (~95%) with different structural features depending of the papaya ripening stage [17]. These different pectin structures might have different host effects by differential modulation of PRR signaling [10,11].

Here we studied PRR signaling of water-soluble fractions of pectins isolated from papaya fruit at different stages of ripening. We focused on attenuation and activating activity of the pectins of TLR and NOD receptors as these have been shown to be the predominant PRRs involved in pectin signaling.

Methods

Plant material

Papayas (*C. papaya* L. cv. 'Golden') were acquired from a producer in Aracruz (Espírito Santo, Brazil) in biological duplicate (2015 and 2016 harvest). The fruit characterization to assure the unripe and ripe classifications was done daily by analyzing fruit respiration (CO_2), ethylene production and pulp firmness [15]**.** The fruits were harvested at color break to one-fourth yellow and stored at ambient temperature until ripe. Five time points were chosen to represent the unripe, intermediate and ripe time points of ripening (1 to 5 five days after harvest - DAH). The total of 6 fruits of each DAH (from two biological replicates) were sliced in small pieces, frozen on N_2 and stored in -80 ºC until the following analysis.

Water-soluble fraction (WSF) extraction

The frozen and sliced papaya were grounded in N_2 and total cell wall was isolated as stated before [16]. From the total cell wall preparation, the WSF was extracted. Briefly, the total cell wall was treated with deionized water under constant magnetic stirring for 20 min at 25 °C and centrifuged (10,000 × g, 20 min, 25 °C) and this step was repeated three times. The WSF were passed through a column with polymyxin B-Agarose to ensure samples were not contaminated with lipopolysaccharides (LPS) as following manufacturer's instructions (Polymyxin B – agarose, Sigma P1411). The LPS-free supernatant (WSF) was lyophilized. Samples were tested for ash content, starch content (Lugol test, and if positive the AA/AMG technique), protein content (microKjeldahl following the AOAC 960.52 method and/or BCA method using Pierce BCA Protein Assay Kit - Thermo Scientific, Waltham, MA, USA) and tested for phenolic compounds (Folin-Ciocalteu test and the SPE-

HPLC-DAD technique if Folin is positive) [18]. The tests all resulted in negligible values, confirming the purity of polysaccharides from more than 99%. WSF samples obtained from the fruits at the first and second DAH correspond to fibers extracted from unripe fruits and the samples were named Un-1-WSF and Un-2-WSF, respectively. WSF sample obtained by the fruits from the third DAH correspond to fibers extracted from the intermediate point fruits and was named I-WSF. WSF samples obtained by the fruits from the fourth and fifth DAH correspond to fibers extracted from the ripe fruits and were named R-1-WSF and R-2-WSF.

Molecular weight distribution

The WSF (3 mg/mL) from different ripening points were analyzed by high-performance size-exclusion chromatography coupled with a refractive index detector (HPSEC-RID) using a 1250 Infinity system (Agilent, Santa Clara, CA). The system was equipped with four PL aquagel-OH columns (60, 50, 40, and 30; 429 300 × 7.5 mm) connected in series. The eluent used was 0.2 M $NaNO_3$/0.02% NaN_3 (0.6 mL/min). The RID temperature was set at 30 °C. Average molecular weight (Mw) was calculated using a standard curve of dextrans (MW 5–1,800 kDa; Sigma-Aldrich (St. Louis, MO, USA)). The void volume (V_o) was the elution time of the heavier molecule (blue dextran; ~1800 kDa), and the elution volume (V_e) was the release time of glucose.

Monosaccharide analysis

High-performance anion-exchange chromatography coupled to a pulsed amperometric detector (HPAEC-PAD) was used for monosaccharide composition analysis [19,20]. Samples (1 mg/mL) were hydrolyzed with 2 M trifluoroacetic acid at 120 °C for 90 min. After the samples were cooled down to room temperature, *t*-butyl alcohol was added, and the mixture was evaporated under N_2 flow. The dried samples were solubilized in water and filtered (0.45 µm) and analyzed in a DX 500 system (Dionex, Sunnyvalle, CA, USA) equipped with a CarboPac PA10 column (250 × 4 mm). Neutral sugars analysis was performed in water (1 mL/min; 40 min). A postcolumn adjustment with 300 mM NaOH was used for detection. Uronic acids analysis was performed in 150 mM NaOH (1 mL/min; 30 min) with a 0–220 nM sodium acetate gradient and postcolumn adjustment with 150 mM. Neutral sugars (arabinose, fucose, galactose, glucose, mannose, rhamnose, and xylose) and uronic acids (galacturonic acid and glucuronic acid) were used as standards.

Determination of degree of methyl-esterification (DM)

The WSF samples (5 mg) was weighed in head-space vials in triplicate. WSF were saponified in duplicate using 1 mL of 0.1M NaOH for 24 h (1 h at 4 °C, followed by 23 h at room temperature). To the WSF blank, 1 mL of water was added. The head-space vials were immediately sealed with a Teflon lined rubber septum. To determine the degree of methyl-esterification (DM) a GC method was used as previously described [21].

Gas chromatography was run on a HS-GC equipped with a flame ionization detector and an automatic injection system. For GC, a Trace GC system (Thermo Scientific, Waltham, MA, USA) equipped with a DB-WAX 30 m × 0.25 mm × 0.25 µm was used. The conditions were as following: helium as carrier gas with a flow rate of 20 mL/min. Column temperature was set at 40 °C for 1,25 min and then programmed to 160 °C at a rate of 20 °C/min. The injector was set at 200 °C and the detector performed at 225 °C. Samples were heated at 50 °C for 10 min in the head-space sampler prior to spitless injection. Two mL of the head-space volatiles was automatically injected in 10 s on the column.

Atomic force microscopy (AFM)

Un-1-WSF and R-2-WSF were diluted in water and sonicated (2.5 µg/mL). The samples were dropped onto freshly cleaved mica, dried in a vacuum at 30 °C for 20 min and maintained in a desiccator until the analysis. An NX-10 Atomic Force Microscope (Park Systems, Suwon, South Korea) in an acrylic glove box was used to obtain the topography images with controlled temperature (~22 °C) and humidity (~3%). AFM images were acquired on tapping mode using an NCHR probe (NanoWorld) with a spring constant of 42 N/m and 320 kHz resonance frequency. The scan speed and scanning resolution were 0.5 Hz and 512 × 512 points, respectively. At least ten images were collected for each sample. Gwyddion 2.47 software (http:// gwyddion.net/) was used to get the images.

Fourier transform infrared (FTIR) attenuated total reflectance (ATR)

The Fourier Transform Infrared (FTIR) spectroscopy was used as a tool to characterize the polysaccharides [22,23]. The Alpha FTIR spectrometer (Bruker Optic, Ettlingen, Germany) equipped with a deuterated triglycine sulfate detector and a single bounce attenuated total reflectance (ATR) accessory (diamond crystal) was used. FTIR–ATR spectra of samples were obtained with a resolution of 4 cm^{-1} and 50 scans.

Reporter cell lines

THP-1 human acute monocytic leukemia reporter and HEK-Blue™ TLR cells were used in the assays (InvivoGen, Toulouse, France). THP-1 MD2-CD14 and THP-1 DefMyD endogenously expresses all human pattern recognition receptors, including all TLRs and express the soluble embryonic alkaline phosphatase (SEAP) gene coupled to the NF-kB/AP-1 promoter. THP-1 MD2-CD14 overexpress CD14 which increases the response to the majority of TLR ligands. THP-1 DefMyD cells are deficient in MyD88 activity turning unable to activate TLR ligands. We used human Embryonic Kidney (HEK 293) blue reporter cell lines with different inserted construct for TLR2, TLR3, TLR4, TLR5, TLR9, NOD1, or NOD2 with all cell lines inserted with the construction for SEAP expression (InvivoGen, Toulouse, France). The activation of TLR and consecutively NF-κB activation will express SEAP. The SEAP is quantified using Quanti-Blue (InvivoGen, Toulouse, France). Specific agonists were used as positive controls for each TLR activation (**Supplementary Table 1**).

THP-1 cell lines were cultured in RPMI1640 culture media (Lonza, Basel, Switzerland) with 10% heat inactivated fetal bovine serum (FBS), L-glutamine (2 mM), HEPES (10 mM), D-glucose (4,5 g/L), sodium pyruvate (10 mM), normocin (100 µg/ml), penicillin/streptomycin (50 µg/mL) and $NaHCO_3$ (1,5 g/L).

HEK cells were cultured in DMEM culture media (Lonza, Basel, Switzerland) with 10% heat inactivated FBS, L-glutamine (2 mM), D-glucose (4,5 g/L), normocin (100 µg/ml) and penicillin/streptomycin (50 µg/mL).

The culture medium of each cell line was supplemented with the selected antibiotic of each cell line (**Supplementary Table 1)**. The WSF were solubilized in DMEM or RPMI1640 at 2 mg/mL, 1 mg/mL and 0.5 mg/mL and cells were treated with these solutions. For the TLR inhibiting/blockage by the polysaccharides, the HEK cells were treated with WSF for 1 h and then, with the specific agonists of each TLR.

After 24 h incubation of cells with the WSF or the other treatments, 20 µL of the cells suspension was added in a new 96-well plate with 180 µL of QuantiBlue solution. After 1 hour of incubation, the plate was read at 650nm in an ELISA plate reader Versa Max (Molecular Devices, Sunnyvale, California, USA).

Statistics

The results were expressed as the mean ± standard deviation (SD). Parametric distribution of data was tested using Shapiro-Wilk normality test. Data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). One-way ANOVA with Tukey's (to assess differences between all groups) or Dunnett's (to assess differences between the control and two or more groups) were used as post hoc tests. Significance was set at $p < 0.001$***, $p < 0.01$** and $p < 0.05$*.

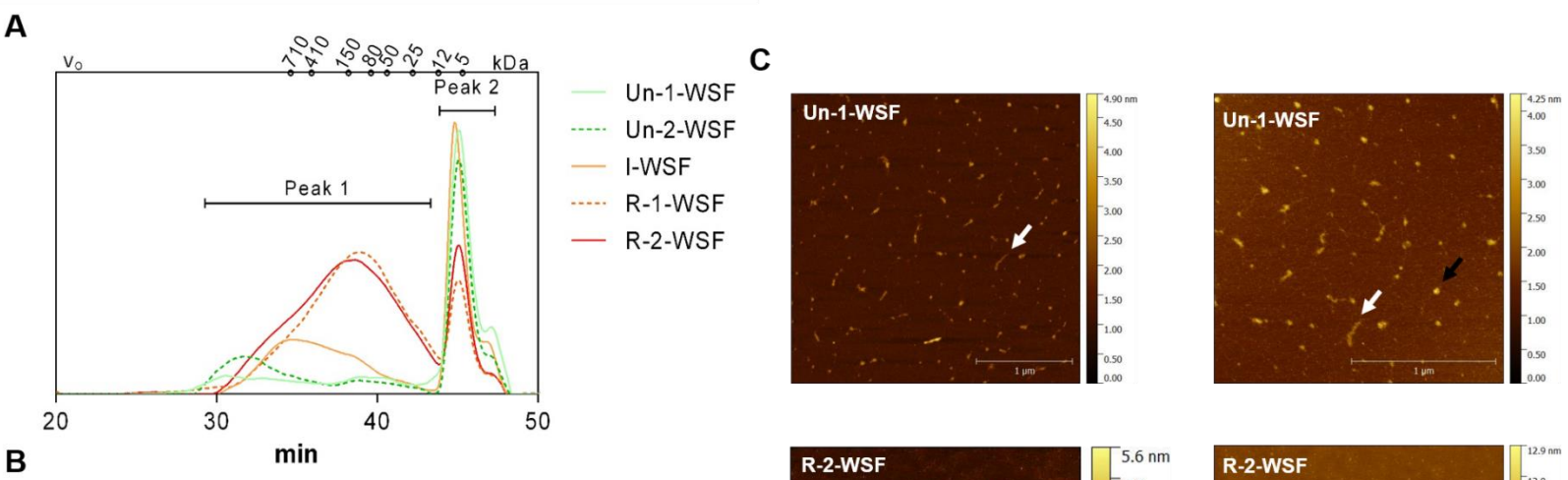
Results

Water-soluble fraction characterization

The WSF samples were obtained from papayas from the first to the fifth days after harvest and the following yield of each fraction is depicted (Un-1-WSF – 1st day after harvest: 0,38 ± 0,02 mg/100g Fresh Weight, Un-2-WSF – 2nd day after harvest: 0,51 ± 0,04 mg/100g FW, I-WSF – 3rd day after harvest: 0,56 ± 0,05 mg/100g FW, R-1-WSF – 4th day after harvest: 0,87 ± 0,05 mg/100g FW, and R-2-WSF – 5th day after harvest: 0,91 ± 0,02 mg/100g FW). The WSF extraction yield increased during papaya ripening as expected [24]. Ash, starch, proteins and phenolic compounds contents were insignificant in the WSF fractions, demonstrating the polysaccharide fractions were highly-purified ones.

Overall, homogeneity and Mw were heterogeneous and with a broad Mw distribution with two distinct populations with the WSF obtained from unripe papayas (Un-1-WSF and Un-2-WSF) having the higher Mw (**Figure 1A, 1B**). The Un-1-WSF and Un-2-WSF were similar between them but different from R-1-WSF and R-2-WSF, these two-last showing lower Mw with similar Mw distribution. The I-WSF possess an intermediary Mw profile compared to other fractions. These statements regarding Mw show that the papaya ripening process hydrolyze pectin while decreasing WSF Mw which will lead to plant cell wall disassembling. The changes in Mw can also be visualized by AFM (**Figure 1C**). In the **Figure 1C** the white arrows represent the linear structure of Un-1-WSF, the black arrows the aggregates with an oval shape and the grey arrow represents the smaller structures of the R-2-WSF. The monosaccharide composition analyses demonstrated that the WSF were mainly composed of galacturonic acid (GalA), a main characteristic of HG portion from pectin structure. The predominant changes during ripening were the enhancement of galacturonic acid (GalA) abundance, while galactose (Gal) and glucose (Glc) were reduced during ripening (**Figure 2**). However, higher amount of Rha was

observed during ripening. When some sugar ratios were compared, a profound difference in the GalA:Rha ratio was observed which was lower in ripe papaya (Table 1) due to high amounts of both GalA and Rha. Gal:Rha and Ara:Rha ratios were also decreased during ripening, which could mean the loss of galactans and arabinogalactans side-chains of RG-I. Taken together this results with our previous study in which papaya WSF was characterized within different timepoints of ripening [17], we suggest that it is occurring an increment of RG-I in WSF from ripe papayas during ripening. Besides that, Ara and Gal decreased during ripening while Rha increased, suggesting the presence of smaller chains of more highly branched RG-I, which is also in accordance with previous study [17], we suggest that is occurring an increment of RG-I in WSF from ripe papayas. Besides that, Ara and Gal decreased during ripening while Rha increased, suggest the presence of smaller chains of more highly branched RG-I, which is also in accordance with previous study [17]. These changes in the WSF profile indicate higher neutral sugars ramifications but with shorter neutral sugar chains in the ripe papaya pectin. The DM was measured in one unripe sample and in two ripe samples. The WSF from unripe (Un-2-WSF) had a DM of 15% and in the ripe samples (R-1-WSF and R-2-WSF) the DM was higher than 40%, demonstrating a proportional increment of methyl-ester groups during ripening due to increase in the WSF yield (**Table 1**). The WSF yield increases during papaya ripening which could indicate an increment of highly esterified pectin. FT-IR spectroscopy was used to characterize the polysaccharide fractions. The frequency band from 1800 to 800 cm^{-1} was selected as the most representative for pectin characterization (**Figure 3**). The pectin structure is assigned by bands in 1740 cm^{-1} (C═O stretching) and 1600-1630 cm^{-1} (COO^- antisymmetric stretching) [22]. The differences in these two bands along the papaya ripening represents the changes in the methyl esterification profile. The 1440 cm^{-1} band represents pectin asymmetric stretching modes vibration of methyl esters [25], 1410 cm^{-1} band the pectin COO^- symmetric stretching [22] and 1235 cm^{-1} band the bending of O─H groups in pyranose ring of pectin [25]. All these bands 1440 cm^{-1}, 1410 cm^{-1}, 1235 cm^{-1} and 832 cm^{-1} (pectin ring vibration [22]) increases as the fruit ripens, demonstrating a possible alteration in ripe pectin structure, with more proportionally galacturonic acid (methyl or not esterified) as it was confirmed by the sugar composition analysis (w/w %, **Figure 2** and **Table 1**).



B

Samples	Molecular weight	
	Mean	sd
Un-1-WSF Peak 1	587	47
Un-1-WSF Peak 2	11	3
Un-2-WSF Peak 1	611	17
Un-2-WSF Peak 2	11	4
I-WSF Peak 1	143	9
I-WSF Peak 2	11	2
R-1-WSF Peak 1	71	17
R-1-WSF Peak 2	11	3
R-2-WSF Peak 1	73	19
R-2-WSF Peak 2	11	1

Figure 1. HPSEC-RID elution profile, molecular weight and AFM images. A) HPSEC elution profile. B) Molecular weight. Values represented by technical triplicate from the biological duplicate. C) Representative topographical AFM images of Un-1-WSF and R-2-WSF. White arrow indicates linear structures, black arrow aggregates and grey arrow the smaller structure from the R-2-WSF. Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction.

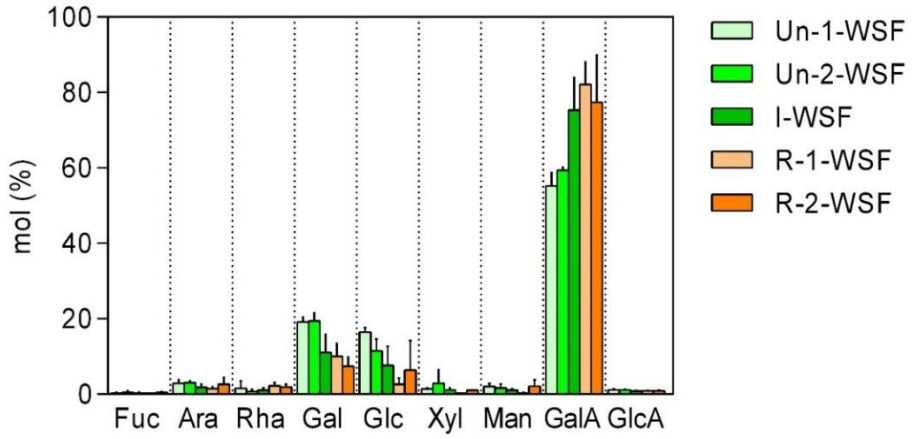


Figure 2. Monosaccharide analysis from papaya water-soluble fractions. WSF: water-soluble fractions. The numbers represent the papaya day (s) after harvested. Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction; Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; GalA: Galacturonic acid; Glc: glucose; GlcA: glucuronic acid. Values represented by technical triplicate from the biological duplicate.

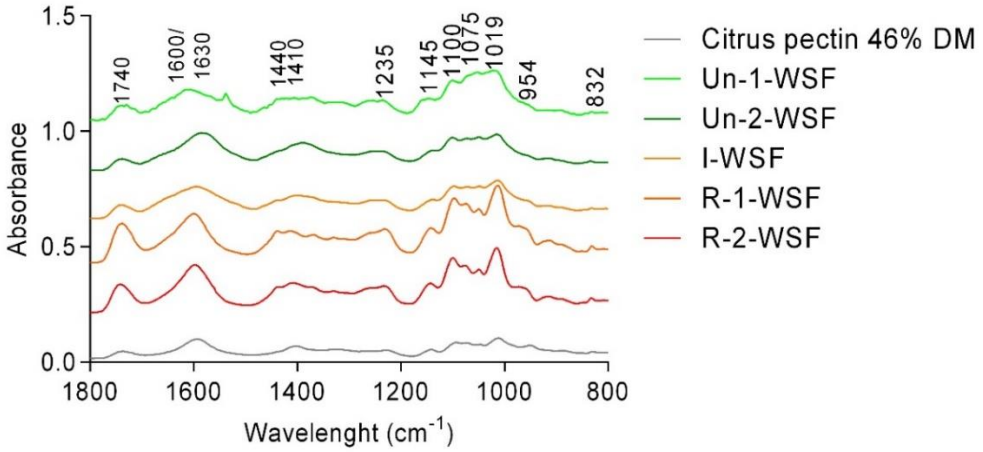


Figure 3. FT-IR spectra of citrus pectin DM 46% and papayas water-soluble fraction (WSF). Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction.

Table 1. Monosaccharide ratios, degree of methyl esterification and monosaccharide composition (w/w %) from papaya water-soluble fractions.

Samples	GalA: Rha	Gal: Rha	Ara: Rha	DM (%)	Fuc	Ara	Rha	Gal	Glc	Xyl	Man	GalA	GlcA
Un-1-WSF	30.3	11.3	2	NE	0.32 ± 0.25	3.64 ± 1.49	1.66 ± 2.04	19.62 ± 1.19	17.00 ± 2.36	1.72 ± 0.65	2.00 ± 0.63	52.95 ± 7.15	1.14 ± 0.21
Un-2-WSF	67.8	23.9	4.5	15.4	0.56 ± 0.53	3.78 ± 0.43	0.87 ± 0.66	20.22 ± 3.31	12.15 ± 4.38	3.31 ± 3.82	1.69 ± 1.13	57.36 ± 6.10	1.05 ± 0.10
I-WSF	60.6	9.6	1.8	NE	0.16 ± 0.1	2.16 ± 1.24	1.21 ± 0.81	11.18 ± 5.80	6.23 ± 3.92	1.47 ± 0.61	1.08 ± 0.30	76.75 ± 16.48	0.81 ± 0.23
R-1-WSF	31.4	4.1	0.7	41.4	0.20 ± 0.08	2.15 ± 0.73	3.02 ± 1.25	12.58 ± 3.28	3.60 ± 1.70	0.38 ± 0.03	0.31 ± 0.23	77.79 ± 6.20	0.87 ± 0.12
R-2-WSF	35.2	3.6	1.5	45.3	0.25 ± 0.31	5.18 ± 3.23	3.8 ± 0.73	12.48 ± 4.87	1.22 ± 0.36	1.86 ± 1.63	0.81 ± 0.40	85.00 ± 2.60	0.83 ± 0.07

NE: not evaluated.
Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction. Rha: rhamnose; Fuc: fucose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; GalA: Galacturonic acid; Glc: glucose; GlcA: glucuronic acid. Values represented by technical triplicate from the biological duplicate.

TLR and NOD signaling of pectin fractions

THP1 MD2-CD14 is a reporter cell line carrying all TLRs coupled to a SEAP reporter gene. Comparison of signaling in this reporter cell with that of a THP-1 cells line with a truncated defective Myd88 (THP1 defMyD88) gene reveals whether a pectin induced SEAP activation is TLR dependent. All papaya WSF significantly increased NF-kB production in THP1 MD2-CD14 when compared with the negative control ($p < 0.0001$; **Figure 4**) while signaling in THP1 defMyD88 was virtually absent. Only in the highest concentration, papaya fractions were able to induce a slight increase in NF-kB production in THP1 defMyD88 reporter cells (p values ranging from 0.05 to 0.0001, **Figure 4**). As this could indicate a concentration dependent activation of other pattern recognition receptors, such as NOD, we decided to also test WSF papaya fractions on NOD signaling in HEK NOD 1 and HEK NOD 2 reporter cells. As shown in **Figure 5**, WSF from ripe papayas induced NOD1 and NOD2 activation in a concentration dependent manner. The pectin from ripe papaya was more profoundly activating NOD2 than NOD1 ($p < 0.05$).

As pectin fractions predominantly activated TLRs in THP1 MD2-CD14 we next determined which specific TLRs are inhibited and/or activated. To this end reporter cell lines expressing either TLR2, TLR3, TLR4, TLR5 or TLR9 were applied (**Figure 6**). We separately studied activating and inhibiting effects of the pectin fractions as following.

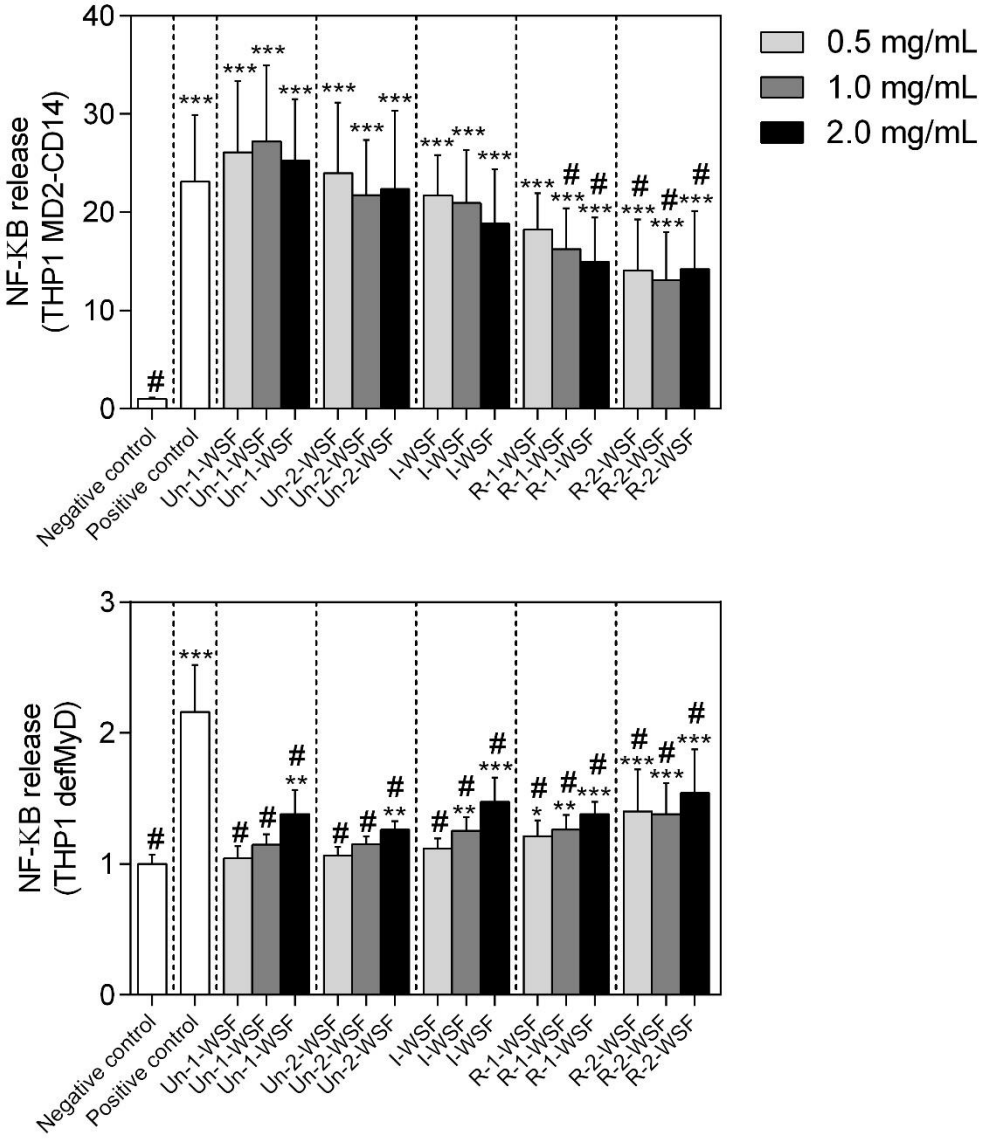


Figure 4. THP1 MD2 CD14 and THP1 defMyD88 reporter cells NF-kB/AP-1 activation after papaya pectin treatments. Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction. According Dunnett's *** p value < 0.0001, ** p value < 0.001, * p value < 0.05 when compared with negative control and # means significantly difference when compared with the positive control.

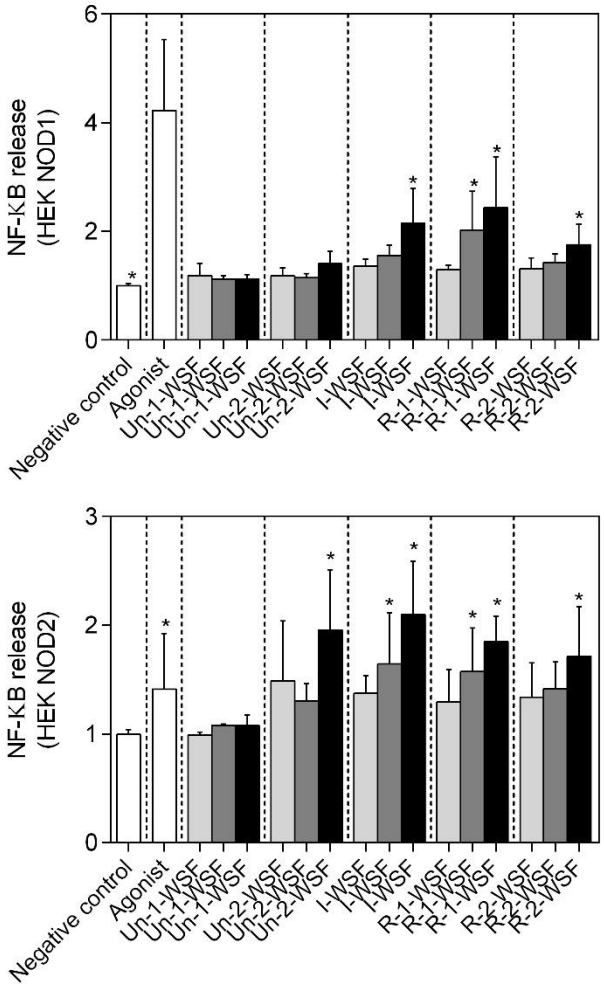


Figure 5. NOD1 and NOD2 reporter cells NF-kB/AP-1 activation after papaya pectin treatments. Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction. According Dunnett's * p value < 0.05 when compared with negative control.

Activating effects

HEK TLR2 cell lines expresses TLR2, TLR1 and TLR6 since signaling of TLR2 activation is dependent of TLR2/TLR6 and TLR1/TLR2 interaction. Activation and/or dimerization of TLR2, TLR2/1 and TLR2/6 was confirmed by stimulation with the specific agonists HKLM, Pam3CSK4 and FSL-1, respectively. We found that all pectins mainly

activated TLR2 and TLR4 ($p < 0.001$). I-WSF, R-1-WSF and R-2-WSF activated TLR3 and TLR5 ($p < 0.001$) and pectin from unripe papayas (Un-1-WSF and Un-2-WSF) did not activate these receptors. I-WSF, R-1-WSF and R-2-WSF significantly activate TLR9 ($p < 0.001$), and only the highest concentration of pectins extracted from unripe papayas (Un-1-WSF and Un-2-WSF) increased TLR9 activation after cell treatment ($p < 0.01$). The unripe fractions Un-1-WSF and Un-2-WSF were not able to activate all TLRs while I-WSF, R-1-WSF and R-2-WSF were able to activate all TLRs. The Un-2-WSF activated TLR 9 in the highest concentration.

Inhibiting effects

As pectins have been reported to have inhibiting effects on TLR signaling in addition to stimulating effects, we also study the possible inhibition. To this end, reporter cells were first treated with papaya WSF for 1 h and then treated with the specific agonists (**Figure 7**). Results for TLR2 (using Pam3CSK4 agonist) and TLR4 were the same as the treatment with only papaya pectin, showing all pectin samples might have in fact activated the receptors. WSF extracted from unripe papaya (Un-1-WSF and Un-1-WSF) inhibited the release of NF-kB after TLR3 induction by the specific agonist, demonstrating a possible irreversible ligation and inhibition of long chain papaya pectin and TLR3. It was observed that the pectin extracted from unripe papaya, both time points (Un-1-WSF and Un-1-WSF), were not able to inhibit the NF-kB release after TLR5 activation by specific agonists, suggesting that those pectins do not interact with TLR5. Only the pectin from the unripe papayas (Un-1-WSF) was able to inhibit TLR9, suggesting this long chain pectin could irreversible interact with TLR9. Another possibility would be a concentration effect, as in unripe WSF has high Mw molecules and in ripe WSF less as it can be seen in HPSEC profile in earlier elution time (31-32 min).

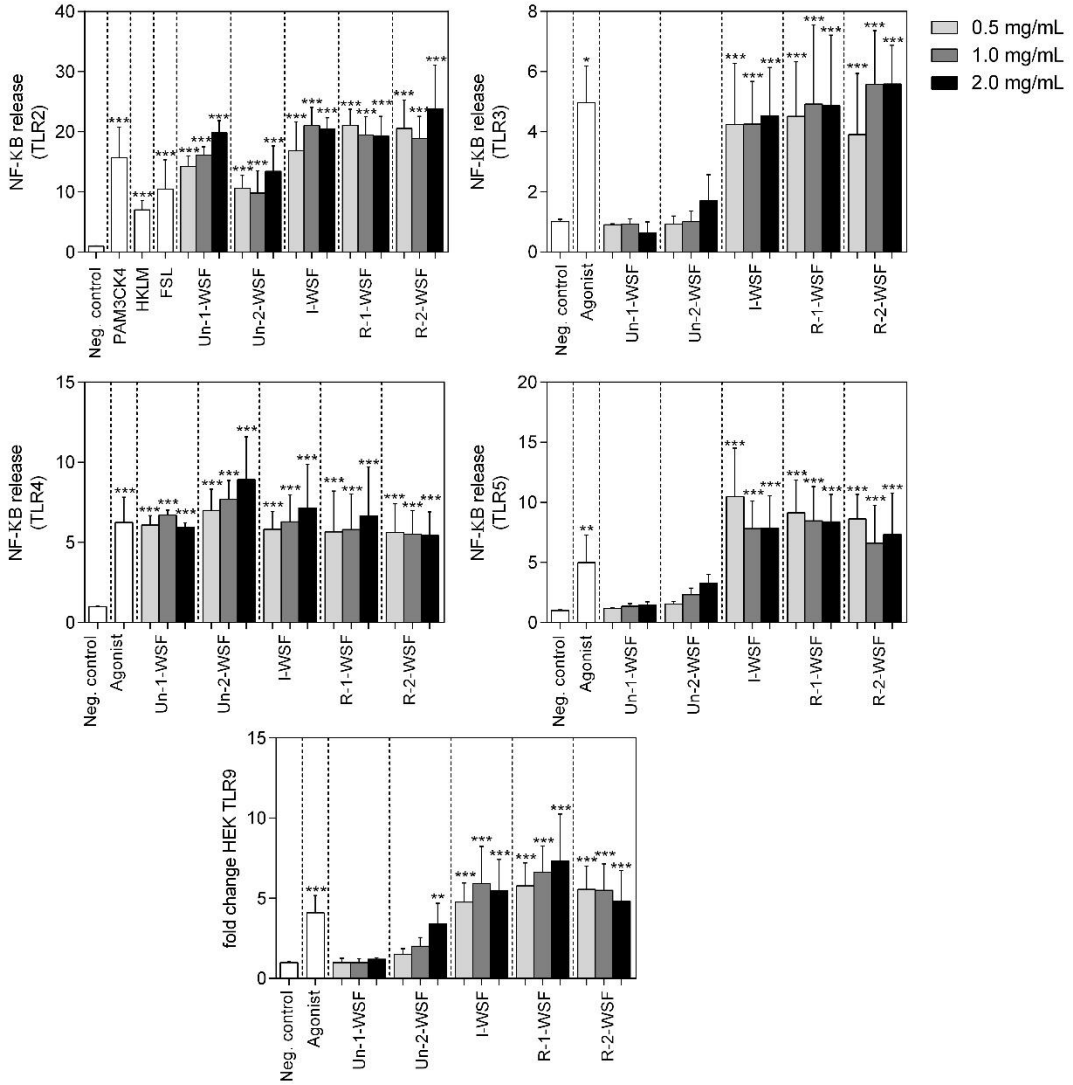


Figure 6. Activation of TLR2, TLR3, TLR4, TLR5 and TLR9 by different papaya pectins. Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction. According Dunnett's test *** p value < 0.0001 and ** p value < 0.001 when compared with negative control.

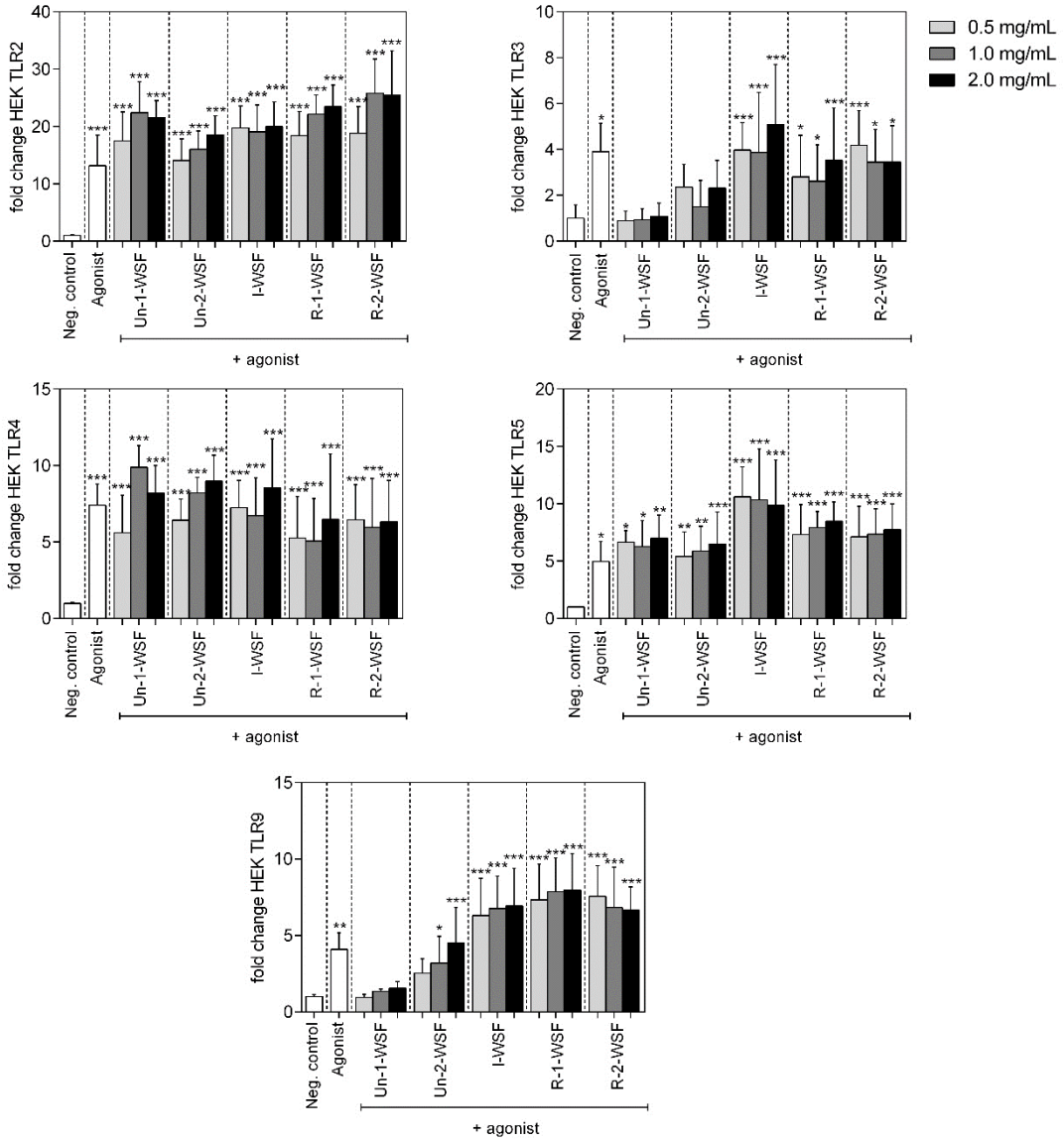


Figure 7. Inhibition of TLR2, TLR3, TLR4, TLR5 and TLR9 by papaya pectin. A agonist of the TLR was applied together with the pectin fraction isolated form papaya Un-1-WSF: unripe - papaya from 1st day after harvest - water-soluble fraction; Un-2-WSF: unripe - papaya from 2nd day after harvest - water-soluble fraction; I-WSF: intermediate ripening time point - papaya from 3rd day after harvest - water-soluble fraction; R-1-WSF: ripe - papaya from 4th day after harvest - water-soluble fraction; R-2-WSF: ripe - papaya from 5th day after harvest - water-soluble fraction. According Dunnett's test *** p value < 0.0001 and ** p value < 0.001 when compared with negative control.

Discussion

Papaya ripening in an enzymatic biochemical driven process that occurs in a short period of time (4-5 days) involving macromolecule mobilization and formation of new unique pectin molecules. Just in three days after harvesting the fruit pulp is completely soft and new pectin molecules are formed due to pectinase time- and ethylene-dependent action [15]. The over-expression of cell wall degrading enzymes, especially polygalacturonases, culminates in pulp softening [16,26]. This massive enzyme action naturally modifies insoluble pectin and releases them in the more soluble fraction in the fruit and hydrolyze the long chains into smaller galacturonans chain [16,17]. The interaction of HG segments derived from pectic polysaccharides with PRR and its concomitant health effects have been described elsewhere [27]; but how papaya pectin in the most soluble polysaccharide fraction (WSF), interacts with PRR, has never been studied. In this study, we extracted the highly-purified water-soluble polysaccharide fraction (WSF) from five time points of papaya ripening (unripe to ripe), characterized the polysaccharides and evaluated whether they differentially interact with TLRs and NODs.

The Water-Soluble Fractions of papaya cell wall consists mainly of homogalacturonan

The structural differences between the WSF derived from unripe, intermediate or ripe papaya stages, are the high amount of GalA and the increasing DM and the Mw distribution of the ripe papayas pulp polysaccharides. The less soluble pectin (only soluble in chelate solvent) represented by long chains of galacturonans (HG) with small portions of de-esterified galacturonans attached to each other by calcium bridges is being released during ripening due to activity of cell degrading enzymes [15]. The main enzymes acting in papaya ripening is polygalactunase which cleave the non-esterified HG parts and by lowering the Mw of the calcium bridges pectins made them more soluble. The enrichment of less soluble pectins into the WSF proportionally increases esterified GalA amount of HG (**Table 1**) and decreases the molar proportion of Gal and Glc throughout ripening as described elsewhere [16]. Even with the decreasing in the overall Mw, the higher Mw do not disappear from riper papaya WSF. This could be due to the continuous solubilization of other lesser soluble cell structures (as alkali soluble structures). This happens not only with papayas [24] but also with other fruits and vegetables [28–30]. Strawberries pectin treated with endo-polygalacturonases and evaluated by AFM profile demonstrated similar results than the stated here with papaya ripening. Bigger linear pectin structures and

agglomerates at first, and after the enzymatic treatment a decrease in the chain length and in the agglomerates [31]. The aggregates seen in AFM image can be polymers complexes held together by intermolecular interactions showing pectin heterogeneity and complexity [32]. Pectin mainly formed by GalA, Ara, Rha, and Gal is the papaya WSF composition, and others neutral sugars like Glc could be derived from more soluble hemicelluloses. The FT-IR analysis confirms that the polysaccharides from WSF extracted from papaya pulps are mainly pectins. As papayas become ripen, a more distinct peak for neutral sugar became apparent. These peaks are related not only with the quantity but also with the position and the degree of substitution of the neutral sugars [33].

THP1 reporter cell lines express all TLRs as well as other PRRs such as NOD1 and NOD2. The activation patterns of the pectins was compared with THP1 defMyD88 reporter cells to determine TLR dependent activation. Our data suggest a possible interaction of papaya pectin with different TLR as well as that of NOD1 and/or NOD2. NOD1 and NOD2 was activated in a concentration dependent way only by pectins derived from the ripe papayas (I-WSF, R-1-WSF, and R-2-WSF). Un-2-WSF seems to be less active, but could also activate NOD2 in the highest concentration as it has already been observed for β2→1-fructans [13].

PRR activation by DF has been mainly described for TLR2 and TLR4 [9,10]. Lemon pectin with DM of 74% activated TLR2 and TLR4 with TLR4 being activated to a lesser extent than TLR2 [10]. However lemon pectin with low DM blocked TLR 2/1 instead of activating the receptor [11]. In human dendritic cells, β-glucans synergistically activate TLR4 and Dectin-1 [34]. β2→1-fructans activated TLR2, while TLR4, TLR5, TLR7, TLR8 were mildly activated in reporter HEK cells [13]. Guar gum can activate TLR2 and dectin-1 reducing the inflammation of small intestine epithelium [35]. Fructooligosaccharides, inulin, galactooligosaccharides, and goat's milk oligosaccharides were reported to be TLR4 ligands in intestinal epithelial cells [36] while the bengkoang fiber stimulated macrophages through TLR4 activation [37]. In our study we observed that even with the DM differences TLR2 and TLR4 were activated after treatment with all papaya pectins. However, TLR3, TLR5 and TLR9 were not activated by pectin derived from the unripe papayas (Un-1-WSF and Un-2-WSF) that have as structural characteristics less methylation and higher Mw molecules. The lower HG structures released in the ripe stage with DM higher than 40% and with higher amounts of GalA seems to interact with the cells receptors in a different way than the less esterified and higher Mw WSF from the

unripe papaya. The lower Mw HG structures released in the ripe stage with DM higher than 40% and with higher amounts of GalA seems to interact with the cells receptors in a different way than the less esterified and higher Mw WSF from the unripe papaya. The changes in neutral sugars during the ripening seem to be related with the decreasing size of the chain ramifications, but with higher amount of Rha which could indicate more ramifications with less sugars linked to it, as also observed elsewhere [17].

The non-activation of the HEK TLR reporter cells could be due to long chain papaya pectins (Un-1-WSF and Un-2-WSF) that are not interacting with the receptors (TLR5) or binding but not activating the receptors (TLR3 and TLR9). The pectin extracted from unripe fruit did not bind to TLR5 since reporter cells treatment carrying this TLR did not show any activation after incubation with these pectin fraction.

The Un-1-WSF and Un-2-WSF treatments inhibited TLR 3 and TLR 9 activation as these pectins specifically contains low-esterified and long chain molecules suggesting they are responsible for inhibition of TLR3 and TLR9. This is corroborated by previous findings demonstrating that low-esterified pectins block TLR 2/1 [11], and highly branched citrus pectin suppressed pro-inflammatory interleukin 6 in RAW 264.7 macrophages stimulated with Pam3CSK4 (ligand for TLR1/2), FSL-1 (ligand for TLR2/6), and CpG-ODN (ligand for TLR9) [38].

Our data also suggest that pectins extracted from ripe papayas, with higher esterification and smaller chains, have the ability to interact and to activate all the studied TLR. It also suggests that pectins from unripe papayas, with lower esterification and longer chains have the ability to interact with TLR3 and TLR9 in an irreversible way blocking the agonist activation. The effects shown in our study cannot be explained by possible contaminations with endotoxins since pectin fractions were pre-treated in an affinity column with polymyxin B-agarose extracting possible endotoxins.

During papaya ripening, profound structural changes in pectin structure occur leading to possible differential biological effects [17,24]. Papaya pectins extracted from fruit pulp at different ripening points could interact with different PRR in a ripening-dependent way. This could represent new biological features of papaya pectins besides the anticancer activities [17,24] possibly creating new and cost-effective approaches to extract pectins with desired structural and biological features from ripe papaya fruits.

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Supplementary file

Supplementary Table 1. Cell culture specifications and agonists used in reporter cells

Reporter cell line (Invivogen)	Selected antibiotic (Invivogen)	Positive controls - agonists (Invivogen)	Cell density for seeding
Thp1-MD2-CD14	Zeocin (uL x mL CM) 100 ug/mL G418 (uL x mL CM) 100 mg/mL	Escherichia coli K12 lipopolysaccharide-HEK ultrapure (LPS) 10 ng/mL	1 x 10^6 cells/mL
Thp1-DefMyD88	Zeocin (µL x mL CM) 100 µg/mL hygro gold (µLx mL CM) 100 µg/mL	L-ala-γ-d-Glu-mDAP (Tri-DAP) 100 µg/mL	2 x 10^6 cells/mL
HEK-hTLR2	HEK-blue (µL x mL CM) 250X	lipopeptide (FSL-1; TLR2/6) 10 µg/mL Heat-killed Listeria monocytogenes (HKLM) 10^7 cells/mL (TLR2) Pam3CysSerLys4 (PAM3CK4; TLR2/1) 10 ng/mL	2.8 x 10^5 cells/mL
HEK-hTLR3	Zeocin (µL x mL CM) 100 µg/mL Blasticidin (µLx mL CM) 30 µg/mL	Polyinosinic–polycytidylic acid high molecular weight (Poly (I:C) HMW) 5 µg/ml	2.8 x 10^5 cells/mL
HEK-hTLR4	HEK-blue (µL x mL CM) 250X	Escherichia coli K12 lipopolysaccharide-HEK ultrapure (LPS) 10 ng/mL	1.4 x 10^5 cells/mL
HEK-hTLR5	Zeocin (µL x mL CM) 100 µg/mL Blasticidin (µLx mL CM) 30 µg/mL	Flagellin from Salmonella typhymurium (Rec-FLA-ST) 10 ng/mL	1.4 x 10^5 cells/mL
HEK-hTLR9	Zeocin (µL x mL CM) 100 µg/mL Blasticidin (µLx mL CM) 10 µg/mL	Class B CpG oligonucleotide (ODN 2006) 0.25 µM	4.5 x 10^5 cells/mL
HEK-NOD1	Zeocin (µL x mL CM) 100 µg/mL Blasticidin (µLx mL CM) 30 µg/mL	L-ala-γ-d-Glu-mDAP (Tri-Dap) 10 µg/mL	2.8 x 10^5 cells/mL
HEK-NOD2	Zeocin (µL x mL CM) 100 µg/mL Blasticidin (µLx mL CM) 30 µg/mL	MurNAc-L-Ala-γ-D-Glu-mDAP (M-TriDAP) 10 µg/mL	1.4 x 10^5 cells/mL

Chapter 6

Chelate-soluble pectin fraction from papaya pulp interacts with galectin-3 and inhibits colon cancer cell proliferation

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Chapter 7

Dietary fiber from unripe and ripe papaya differentially regulate microbiota composition: Evidence from an *in vitro* colonic fermentation study

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Abstract

Dietary fibers (DF) consumption is recommended because of the healthy and protective effects in decreasing the risk of diseases, such as colon cancer. DF have complexes structures that is determinant for their biological effects that includes the indirect effects of SCFA, and the effects of DF on the modulation of the microbiota profile. In the present study, we explore the biological effects of DF from unripe and ripe papaya using colonic *in vitro* fermentation. These effects include the utilization of the DF by the fecal inoculum, the production of SCFA, and the microbiota profile that was characterized by 16S sequencing. Results shown that DF from unripe and ripe papaya induces a similar production of SCFA, characterized by increased levels of acetate, propionate and butyrate, compared to apple pectin and lactulose, which were also used as fermentation substrates. Besides the similar results concerning the SCFA, the DF utilization by the bacteria showed a distinct profile. The DF from unripe papayas had a slow bacteria utilization than the DF from ripe papayas, and the latter had a faster oligosaccharide degradation by the microorganisms. Furthermore, weighted UniFrac distances matrix analysis revealed significant changes in the bacteria profile after 24 h of fermentation compared to the initial time of fermentation (0 h), the differences were between both the DF from unripe and ripe papayas and the apple pectin and lactulose at 24 h of fermentation. Notably, although DF from unripe and ripe papaya induces similar results concerning the levels of SCFA, the colonic fermentation of these DF induces distinct changes in the bacteria profile. Fermentation of DF from unripe papaya increased the abundance of *Clostridiaceae 02d06*, *Coprabacilus*, *Bulleidia* and *Slackia* genera, and both unripe and ripe papayas DF showed an enhancement of *Clostridium* and *Bacteroides*. Therefore, the DF utilization from papaya by the bacteria is dependent of the carbohydrate structure, resulting in distinct changes in bacteria profile. However, besides their differences in the carbohydrate structure and therefore on the induction of a distinct bacterial profile, DF from unripe and ripe papaya similarly increased the production of acetate, propionate and butyrate, demonstrating the possible benefits of ingesting both types of DF.

Key-words: *in vitro* fermentation; dietary fiber; pectin; papaya; microbiota.

Abbreviations: Ara, arabinose; DF, dietary fibers; Gal, galactose; GalA, galacturonic acid; Glc, glucose; HG, homogalacturonan; RGI, rhamnogalacturonan I; SCFA, short chain fatty acids; Xyl, xylose.

Introduction

Dietary fibers (DF) consumption has been associated with several health benefits including the reducing both postprandial glucose levels and the reabsorption of bile acids [1], as well increasing satiety and stool bulk [2] and effects resulting from both the direct interaction with cells (intestinal epithelial cells and immune cells) [3] and fermentation-related effects [4]. The DF fermentation process lead to the production of substrates including short chain fatty acids (SCFA) such as acetate, propionate and butyrate [5,6]. The increase in SCFA levels help in lowering the intestinal pH thus favoring the environment for healthy associated bacteria, thereby reducing the establishment of pathogenic bacteria [7]. Butyrate is used as a primary source of energy for intestinal epithelial cells, stimulating both the growth of these cells and the production of cytokines that act protecting the epithelial integrity [8,9]. Acetate is usually the SCFA produced in higher amounts during DF fermentation [10] and is absorbed and metabolized in muscle, kidney, heart, and brain tissues [11]. Propionate is metabolized in the liver, and appears to suppress cholesterol synthesis in addition to enhance the antiproliferative properties of butyrate in cancer cells [12].

Some intestinal bacteria have a repertoire of carbohydrate-active enzymes that can hydrolyze specific glycosidic linkages from DF [13]. Thus, the knowledge of DF structures are crucial to establish a relationship between the effect of a specific DF structure and changes in the fermentation profile by the gut microbiota. The fruit cell wall is a complex structure composed mainly by cellulose, hemicellulose and pectin, which are composed mostly by DF [14]. Cellulose is a structure composed by 1,4-β-D-glucose (Glc) residues [15] and hemicellulose is composed by a range of structural polymers such as xyloglucan [16], which consist in a backbone of 1,4-β-D-Glc residues such as cellulose, but containing 1,6-α-D-xylose (Xyl) side chains [17]. The most abundant structure in pectin is homogalacturonan (HG), which is a linear structure comprised of α-1,4-α-D-galacturonic acid (GalA) residues with varying degrees of acetyl and methyl esterification. Pectin also contain ramified structures such as rhamnogalacturonan I (RGI), which have a backbone with repeating units of GalA (1,4-α-D-GalpA) and rhamnose (1,2-α-L-Rhap), usually containing side chains of arabinose (Ara) and galactose (Gal) [18,19].

Papaya (*Carica* papaya L.) is a climacteric fruit with a fast ripening after harvesting with a concomitant change in cell wall structure that leads also to a fast pulp softening. Just around 3 to 5 days after harvesting green papayas, the fruit pulp become completely soft and ready for human consumption [20]. Polygalacturonases are ethylene-controlled enzymes that act hydrolyzing papaya cell wall in just a few days, making the papaya pulp soft by turning the insoluble pectin into water-soluble pectin by decreasing the molecular weight of these less soluble pectin [21,22]. Despite the structural changes that occur in papaya cell wall during ripening, it is unknown whether these differences in DF structure of unripe and ripe papayas influences their fermentation profile. As DF are not digested by the human enzymes and reach the colon where they are fermented by the gut microbiota, it is important not just know their whole structure and utilization by the microbiota but also the growth profile of the bacteria that are being favored. Thus, in this study, we used an *in vitro* colonic fermentation approach to explore the utilization of DF from unripe and ripe papaya by intestinal bacteria, as well as the effects of these DF on SCFA production and modulation of the bacteria profile by 16S sequencing.

Material and methods

Plant material

Papaya (*Carica papaya* L. cv. "Golden") from two biological replicates were acquired from a producer in Aracruz (Espírito Santo, Brazil; 19°47′42.2″S, 40°19′09.2″W) as described in Prado et al. (2018), in which the same fruit sampling was used [23].

Dietary fiber extraction from papayas

The frozen papaya pulp was ground in N_2 and incubated three times in boiling chloroform:methanol (1:1; 15 min) in order to get rid of the proteins, lipids and pigments. The solid residue was further washed in boiling 80% (three times) ethanol to extract the remaining soluble sugars and then in acetone. After washing, the residue was dried at ambient temperature to obtain the total cell wall (DF) of papaya pulp.

Batch colonic *in vitro* fermentation

Feces of three volunteers were used to make a pool, which was used to prepare the fermentation inoculum. For that, three health volunteers with no history of antibiotics usage on the last three months were recruited after Ethics Committee approval (School of Pharmaceutical Sciences, University of São Paulo; Number #1.089.446). The feces were collected in a sterile container and immediately put on ice until the sample delivery, which was done at the same day. The *in vitro* fermentation assay was based on previously described methods [24,25]. Briefly, after receiving the fecal samples, the inoculum was immediately prepared.

After pooling the feces, they were diluted in anaerobic and sterile 0.9% NaCl solution in a ratio of 1:6 (w/v). The material was homogenized and filtered (Miracloth, EMD Millipore), resulting in the inoculum, which was put in a CO_2-filled container for anaerobic condition maintenance. The fermentation medium prepared contained basal solution (76%), vitamins/phosphate buffer solution (1%), bicarbonate buffer (1%) and reducing agent (4%) as described in Williams et al. (2005). All bottles and containers used were sterile and the bottles with the medium and the inoculum were continually flushed with CO_2, as described in the original methods.

The steps of the *in vitro* fermentation assay are summarized in **Figure 1**. The carbohydrates used as substrate were unripe papayas DF, ripe papayas DF, apple pectin (Sigma, 76282), and lactulose (Sigma, L7877). Each fermentation bottle was added 100 mg of a specific carbohydrate, 24 mL of fermentation medium and 1.5 mL of the inoculum. The CO_2 was flushed inside the screw capped bottles and they were incubated in a water bath at 39 °C. A blank control was used, without the addition of any kind of DF. A bottle with just the fermentation medium was also used as negative control of medium sterility. A duplicate of each treatment was used for the analysis each fermentation time (0 h, 8 h, 16 h and 24 h).

The pH and pressure were evaluated at each fermentation time point as an indicator that fermentation is occurring. The fermentation content was gently centrifugated and aliquots of the supernatant were separated for the analysis of SCFA and carbohydrates (Monosaccharide analysis, homogeneity and average molecular size, and oligosaccharide

analysis). The precipitates were immediately frozen using N2 and storage at –80 ºC until DNA analysis.

Monosaccharide analysis

High-performance anion-exchange chromatography coupled with a pulse amperometric detector (HPAEC-PAD) was used to analyze the monosaccharide composition [26,27]. DF from unripe and ripe papayas (1 mg) were hydrolyzed using 2 M trifluoroacetic acid at 120 °C for 90 min. *T*-butyl alcohol was added in the mixture and the hydrolysate was evaporated under N_2 flow, solubilized in water, and filtered. An DX 500 system (Dionex) equipped with a CarboPac PA10 column (250 × 4 mm; Dionex) was used to analyze the samples. For the analysis of neutral sugars, the eluent was 150 mM NaOH (1 mL/min; 30 min), and a post column adjustment with 300 mM NaOH was used. For the uronic acid analysis, the same system was used but a 0–220 mM sodium acetate was used as gradient eluent. Neutral sugars and uronic acids (Sigma) were used as standards.

Homogeneity and average molecular size

Supernatant (1 mL) from the fermentation time points were filtered (0.22 µm) and analyzed through high-performance size-exclusion chromatography coupled with a refractive index detector (HPSEC-RID). In a 1250 Infinity system (Agilent, Santa Clara, CA). The system was equipped with four PL aquagel-OH columns (60, 50, 40, and 30; 429,300 × 7.5 mm; Agilent) connected in tandem. The eluent was 0.2 M NaNO3/0.02% NaN3 (0.6 mL/min), and the RID temperature was set at 30 °C. An external curve of dextran (MW 5–1800 kDa; Sigma) was used as size standards.

Oligosaccharide analysis

Supernatants (1 mL) from the fermentation time points were analyzed using a DX 500 HPAEC-PAD system (Dionex) as described by Jonathan et al. (2012). The samples were filtered (0.22 µm) and analyzed in a CarboPac PA- 1 column (2 mm × 250 mm; Dionex). Oligomers derived from neutral sugars were eluted (0.3 mL/min) with a linear gradient of 0.02–0.05 M NaOH for 3 min and 0.05–0.075 M NaOH for 10 min, followed by isocratic elution of 0.1 M NaOH for 2 min. Oligomers derived from uronic acids were

eluted with a gradient of 0–1 M NaOAc in 0.1 M NaOH for 50 min. Finally, the column was washed with 1 M NaOAc in 0.1 M NaOH for 7 min followed by 0.1 M NaOH for 3 min. Equilibration was done by eluting 0.02 M NaOH for 20 min.

Bacterial DNA extraction and 16 s sequencing

The fermentation precipitate containing the inoculum bacteria had the DNA extracted using the kit PSP® Spin Stool DNA Kit (Stratec Molecular) and lysis beads (Lysing matrix E, MP Biomedicals) following the manufacturer's instructions. The 16S rRNA gene was amplified using primer pairs, BSF8 (27F) e BSR357 (338R), targeting the V1 and V2 hypervariable region. AMPure XP (Beckman Coulter) was used to purify the PCR products, according to the manufacturer's instructions. Amplicons were generated according to the Illumina MiSeq 16S Metagenomic Sequencing Library. Samples were prepared using the Nextera XT Index kit (Illumina) and the quality of libraries was assessed and quantified using Bioanalyzer (Agilent) and Qubit (Life Technologies), respectively. The 16S amplicon sequencing was performed at the Core Facility for Scientific Research of the University of São Paulo (CEFAP/ICB-USP). Prepared libraries were pooled and then sequenced in a paired-end 2x300bp format on an Illumina MiSeq platform. Quantitative Insights Into Microbial Ecology (Qiime) analysis was done by Tau GC Bioinformatics. Paired end sequence experiments had read pairs joined with PEAR (v0.9.10) using default settings (p:0.01,v:10,m:0,n:50,t:1,q:0,u:1,g:1,e,s:2,b:33,c:40). Sequence analysis was done using the Qiime package version 1.9.1 and enclosed reference Greengenes database from August 2013. OTU picking and taxonomic assignment was done using pick_de_novo_otus.py script with default parameters, i.e.: 97% sequence identity, first (seed) representative sequence and consensus taxonomy assigned using uclust (v1.2.22q); alignment with PyNAST (v1.2.2) removed all gaps positions and mask non-conserved positions; representative OTU sequence tree building with FastTree (v2.1.3). The unrarefied Weighted UniFrac distance matrices were used. OTU networks were generated using Qiime´s make_otu_network.py script. Taxonomy summaries figures were done without count normalization (collapse_samples.py). Alpha diversity was done with rarefaction (alpha_rarefaction.py) using 10 steps of both the median number of sequences between samples and 5000 sequences. Beta diversity was calculated with jackknifing for both weighted and unweighted UniFrac (v1.5.3) (jackknifed_beta_diversity.py). The abundance percentage and heatmaps were made using the OTU table, taxonomy table and

the metadata file with MicrobiomeAnalyst tools (https://www.microbiomeanalyst.ca/faces/home.xhtml).

Statistics

The results were expressed as the mean ± standard deviation (SD), and the significance was set at $p < 0.05$. Data were analyzed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). One-way ANOVA with Tukey's (to assess differences among all groups) was used as post hoc test.

Results and Discussion

The colonic *in vitro* fermentation was performed at 0, 8, 16 and 24 h using DF extracted and isolated from unripe and ripe papayas as substrate. Furthermore, a commercial apple pectin was used as a standard type of DF, and lactulose as a disaccharide that is completely fermentable. A control blank with inoculum without any DF was used in order to visualize any alteration from the possible fermentable substrates that come from the volunteers feces, and another control with only medium was also used to demonstrate the sterility of the inoculum, that resulted in no fermentation and no alteration in any studied parameter, such as medium pH and flask pressure.

The DF from unripe and ripe papaya differs in the amount and composition of their soluble DF. The main changes in papaya cell wall (mainly formed by DF) during ripening is caused by the action of polygalacturonases, a pectinase that are produced after ethylene triggering during the fruit climacteric with a huge impact on the degradation of papaya cell wall [21,22]. The papaya fruit enzymes act not just mobilizing the insoluble pectins to water-soluble pectins but also degrading the soluble pectins into a smaller molecular size [21,22,26,28]; therefore, the main change in papayas DF during the ripening is in the pectin structure. It can be easily confirmed by measuring the yield of the DF that is water-soluble obtained from unripe and ripe papayas. Previous study using the same sampling revealed that the yield of the most soluble pectin (water-soluble fraction) extracted from unripe papaya was 34%, whereas the yield of the same fraction from ripe papaya was 57% [23]. The difference on DF solubility and pectin composition is probably the reason why the DF from unripe or ripe papayas present different effects on fermentation describe herein.

Besides that, using the whole DF and not just the most soluble part, it was proposed here to determine a most reliable simulation of human digestion, and how these DF from papaya pulp could be bio-transformed by the intestinal bacteria.

The monosaccharide composition of DF from unripe and ripe papaya showed that the latter has lower amounts of GalA, Gal and Glc, and increased Rha levels (**Figure 2**). These monosaccharides residues are usually associated with pectin structure, that in papaya is formally mostly by HG and RGI. The increased amount of Rha indicates the presence of more ramified RGI, even with less extend branching – as Gal and Ara are diminished – thereby indicating that arabinans and arabinogalactans are probably smaller than in unripe papayas DF [28]. Other components of the fruit cell wall are also present in papaya pulp, as cellulose that is formed by Glc residues, and hemicellulose that is composed by Xyl, Glc, Man and/or Gal residues.

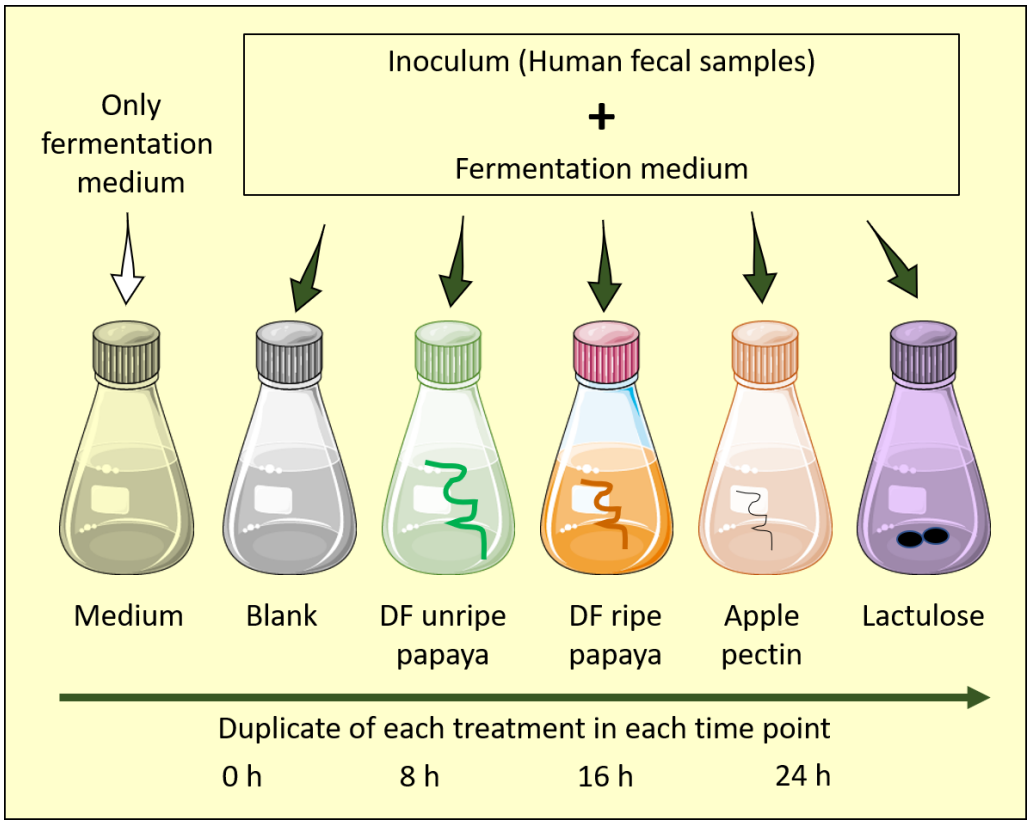

Figure 1. Schematic representation of the *in vitro* batch colonic fermentation designed for this study.

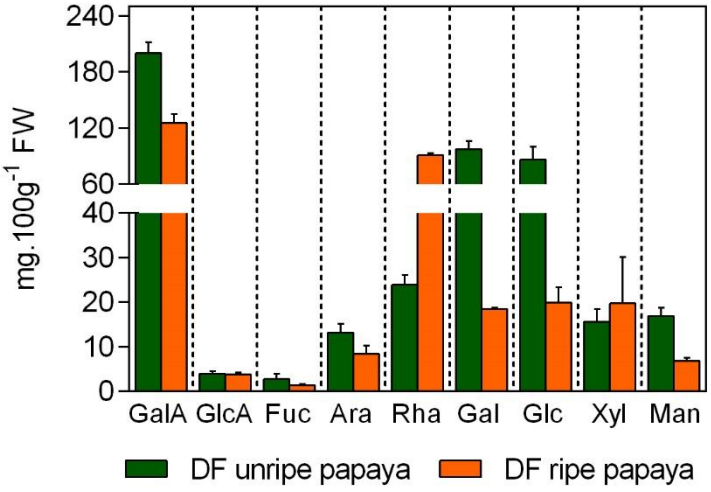

Figure 2. Monosaccharide composition of DF from unripe and ripe papaya.

The pH and pressure were used to indicate that the fermentation is normally occurring in the samples. According to **Figure 3A**, DF from unripe papaya showed a rapid increase in pressure and reach pressure levels similar to that of lactulose at 24 h. Lactulose is largely referred as a control of fermentation and its pH after fermentation is commonly associated to the lowest values [29–31], as we observed in our experiments (**Figure 3B**). The resulted pH of both DF from unripe and ripe papayas and the apple pectin showed lower values than the blank (only the inoculum). The pH of 0 h and 24 h were 7.61 and 6.11 for the blank, 7.38 and 5.51 for lactulose group, 7.04 and 6.05 for apple pectin group, 7.73 and 6.09 for DF from unripe papaya, and 7.8; and 6.13 for ripe papya. Therefore, the differences between the pH on the beginning and the final of fermentation were 1.50, 1.87, 0.98, 1.64 and 1.67 for blank, lactulose, apple pectin, and DF from unripe and ripe papaya, respectively. Thus, although lactulose have the highest reduction in pH the value during the fermentation, the DF from both unripe and ripe papaya also showed a higher decrease compared to the blank and to apple pectin.

In accordance with pH levels, the SCFA production were observed as the highest levels after 24 h of fermentation (**Figure 4**). Acetate showed the greatest accumulation mainly for DF from unripe and ripe papayas and for lactulose. Acetate is normally the main product of DF fermentations [32,33], and is associated with effects on lipid metabolism, whereas propionate is associated with reduced synthesis of fatty acids in both liver and

plasma [34]. Butyrate was significantly increased in DF from unripe and ripe papayas compared to other treatments. Butyrate is related to maintain integrity of intestinal cells and reduces pro-inflammatory signaling [35,36]. The *in vitro* fermentation of citrus pectin also increase acetate and butyrate levels [37]. Recently, it was shown that inulin, fructooligosaccharides, native pectin from citrus (10–12 kDa and 100–800 kDa) and sunflower (100–800 kDa), and modified pectin from sunflower (12.5 kDa) and artichole (80–300 kDa) were used as substrate for *in vitro* fermentation, and results suggests that neither the degree of methoxylation nor the molecular weight of these pectin influences SCFA production, as all samples showed similar levels of acetate, propionate and butyrate [38]. One interesting question is why the pH of lactulose was the lowest one, but the sum of the SCFA was not the highest one. This could be explained by the fact that the fermentation of lactulose will produce high amounts of lactic acid with low transformation in acetate thus lowering the pH [30]. We did not perform this analysis since pectin fermentation, in general, did not result in high amounts of lactic acid [39], and the beneficial effects for human health is related to the SCFA produced and studied in this work, especially the butyrate.

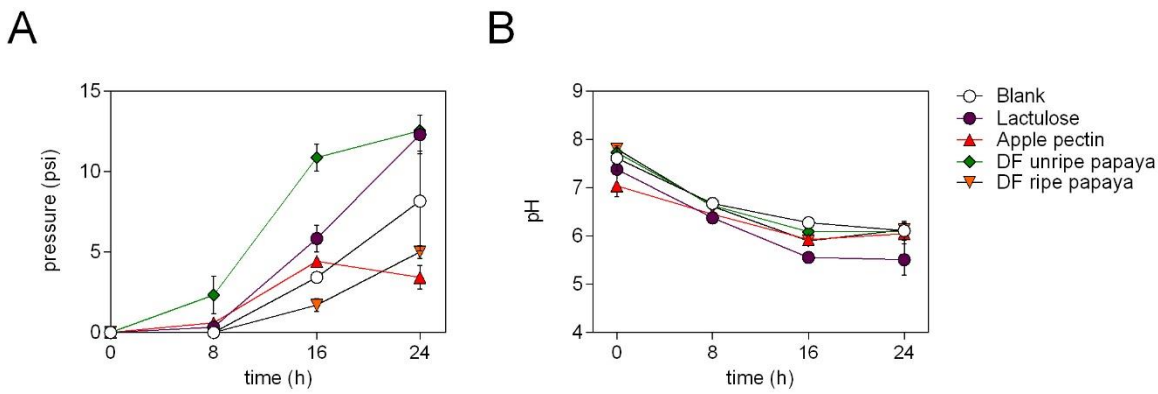

Figure 3. Pressure and pH of *in vitro* colonic fermentation time points.

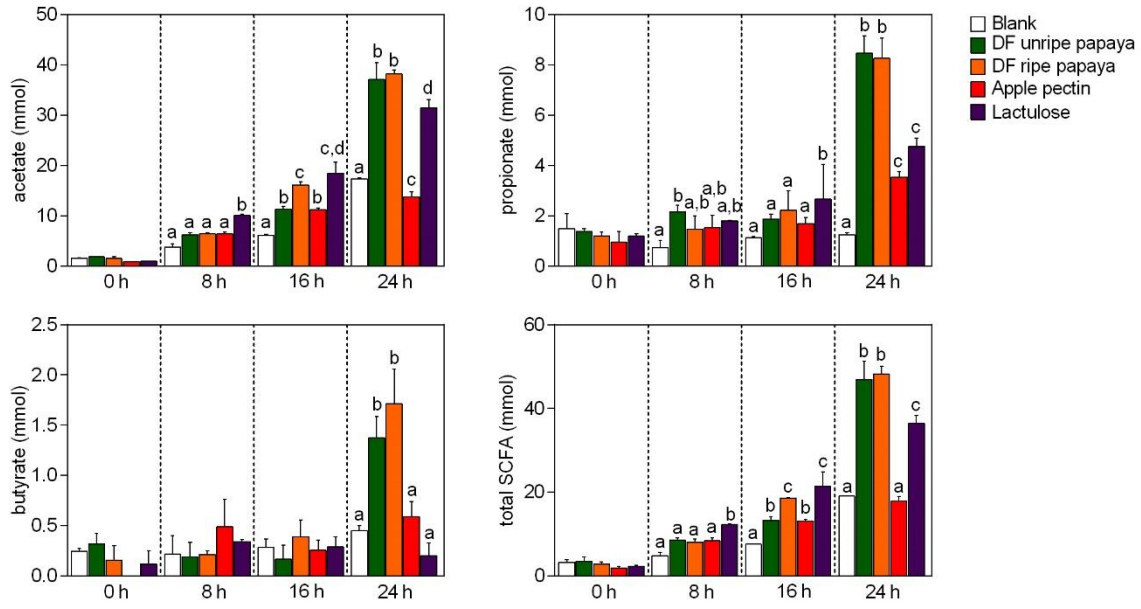

Figure 4. Short chain fatty acid production: acetate, propionate, butyrate and total SCFA (the sum of acetate, propionate and butyrate). Data were shown as mean ± SD. Tukey's test (*p < 0.05) was performed. Different letters represent significant differences between the treatments of each time.

The carbohydrates degradation caused by fermentation were evaluated by homogeneity and average molecular weight and oligosaccharide analysis. In the earliest fermentation time-points it can be seen higher molecular weight structures for unripe and ripe papayas DF, and for apple pectin, with peaks eluted around 5 and 150 kDa of equivalent dextran (**Figure 5**). The blank (without DF as substrate) do not have signals detected equivalent to 5 and 150 kDa of equivalent dextran molecular weight (**Supplementary Figure S1**). Peaks eluted around 59 – 63 min could be due to fermentation medium interference, as seen in **Figure S1** with only the medium being detected in HPSEC. However, on the later times of fermentation it is possible to see a higher peak eluted around 63 min for ripe papayas DF and lactulose, indicating that ripe papayas DF fermentation have a different pattern than DF from unripe papayas. The peaks eluted after 65 min were considered interference of lower molecules as monosaccharide and organic acids [40]. The smaller fermentation products can be better visualized and separated through the oligosaccharide analysis (**Figure 6**). The peaks that eluted before 5 min were considered as monosaccharides and interferences, but after 5 min a distinct oligosaccharide patterns can

be observed to the samples. The blank, which contains only the fermentation medium showed lower signals. Apple pectin had increased detection of polymers, and lactulose showed less signals as expected for a disaccharide. DF from unripe and ripe papaya did not showed signals at 0 h of fermentation, and peaks derived from ripe papaya appeared at early fermentation times compared with DF from unripe papayas. At 16 h of fermentation the DF from ripe papaya presented higher signal compared to DF from unripe papaya, and the peaks of the first seem disappear at 24 h of fermentation, indicating a higher consumption of oligosaccharides after 24 h of the fermentation. Others studies showed that different lengths of isomalto/malto-polysaccharides showed distinct utilization and fermentation ratio [40]. Ensiled chicory root pulp (more soluble) had higher utilization by fermentation than native chicory root pulp (less soluble). These differences appear to be associated with an increased levels of soluble pectin (HG and RGI) and probably to a more open structure that is more accessible for the enzymatic degradation [41]. *In vitro* fermentation of linear arabino-oligosaccharides and debranched (linear) sugar beet arabinans showed that the former were slowly fermented than the last [42]. Thus, the abovementioned studies indicate that changes in carbohydrate structure (size and composition) influences the utilization of DF by the microbiota, and possibly changes in the bacteria profile.

The bacteria profile was evaluated throughout the distance between the bacterial profile from blank (0 h fermentation) to the treatments at 24 h, calculated using Weighted UniFrac distances matrix (**Figure 6A**). The closer to the number 0 on y axis means that the sample is more similar to the blank (0 h), and the higher the value on y axis means that the sample is more different to the blank (0 h). Lactulose and apple pectin on 24 h of fermentation were significantly different from DF extracted from unripe and ripe papayas. No statistical difference was observed between the DF from unripe and ripe papayas, meaning that both are more distant of blank 0 h than the other substrates, but not necessarily meaning that these DF have the same changes in bacteria profile. To evaluate the types of bacteria that could have changed over the fermentation, a percentage of phylum and family abundance is shown in **Figure 6 B and Figure C**, respectively. The phylum data suggests that at 8 h of fermentation Bacteroidetes decrease and the Proteobacteria increase. The enhancement of Proteobacteria at 8 h can be explained by the initial process of *in vitro* fermentation that inevitable has more oxygen, even though the anaerobic condition was provided. At 24 h of fermentation the Bacteroidetes increase again, and for DF from unripe and ripe papaya the Bacteroidetes abundance seem to be equal or higher at 24 h compared

to 0 h. The bacteria composition of the Bacteroidetes Phyla at 0 h is mainly composed by *Prevotellacaeae* and *Paraprevotellaceae*, and at 24 h for DF from ripe papaya the *Bacteroidaceae* family is prevalent.

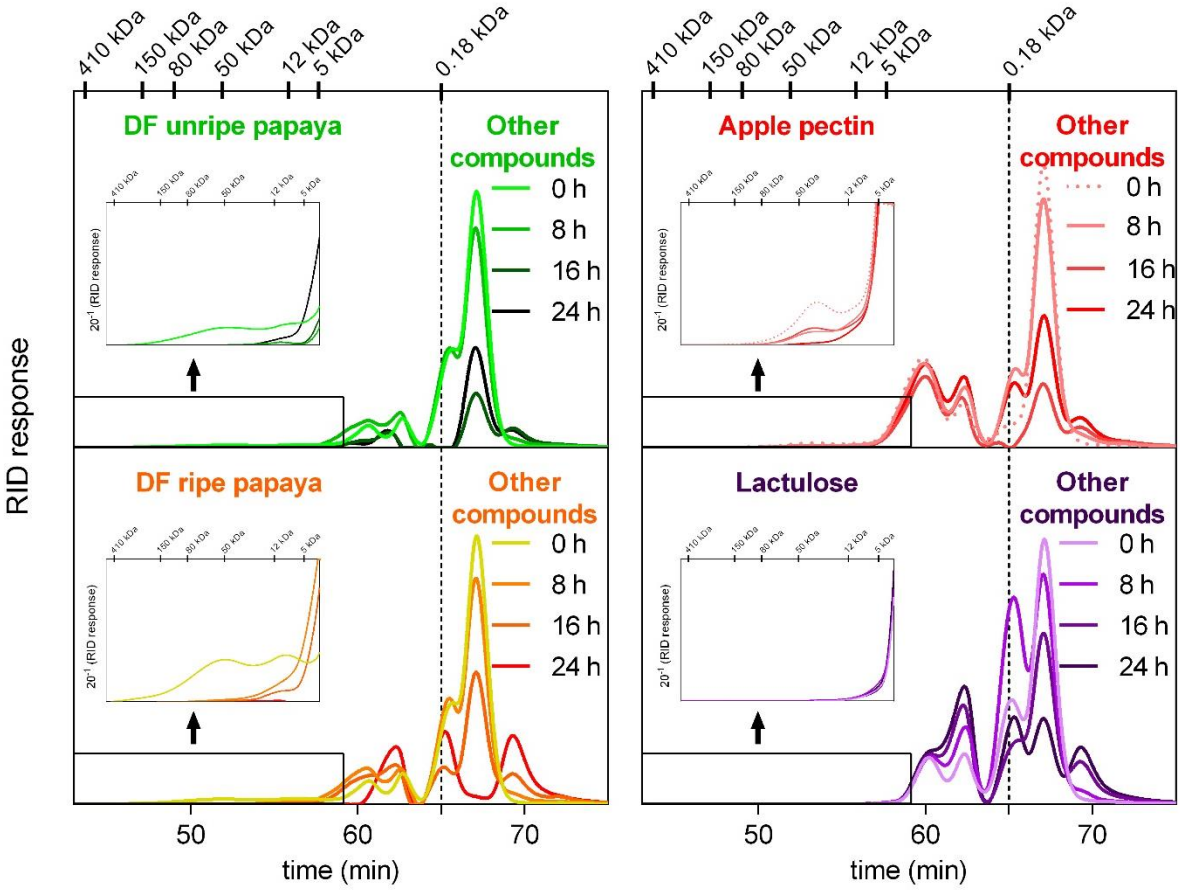

Figure 5. HPSEC of colonic *in vitro* fermentation aliquots. The inside boxes represent a zoom on the marked area. Above to the graphs are indicated the standards dextran sizes and glucose (0.18 kDa).

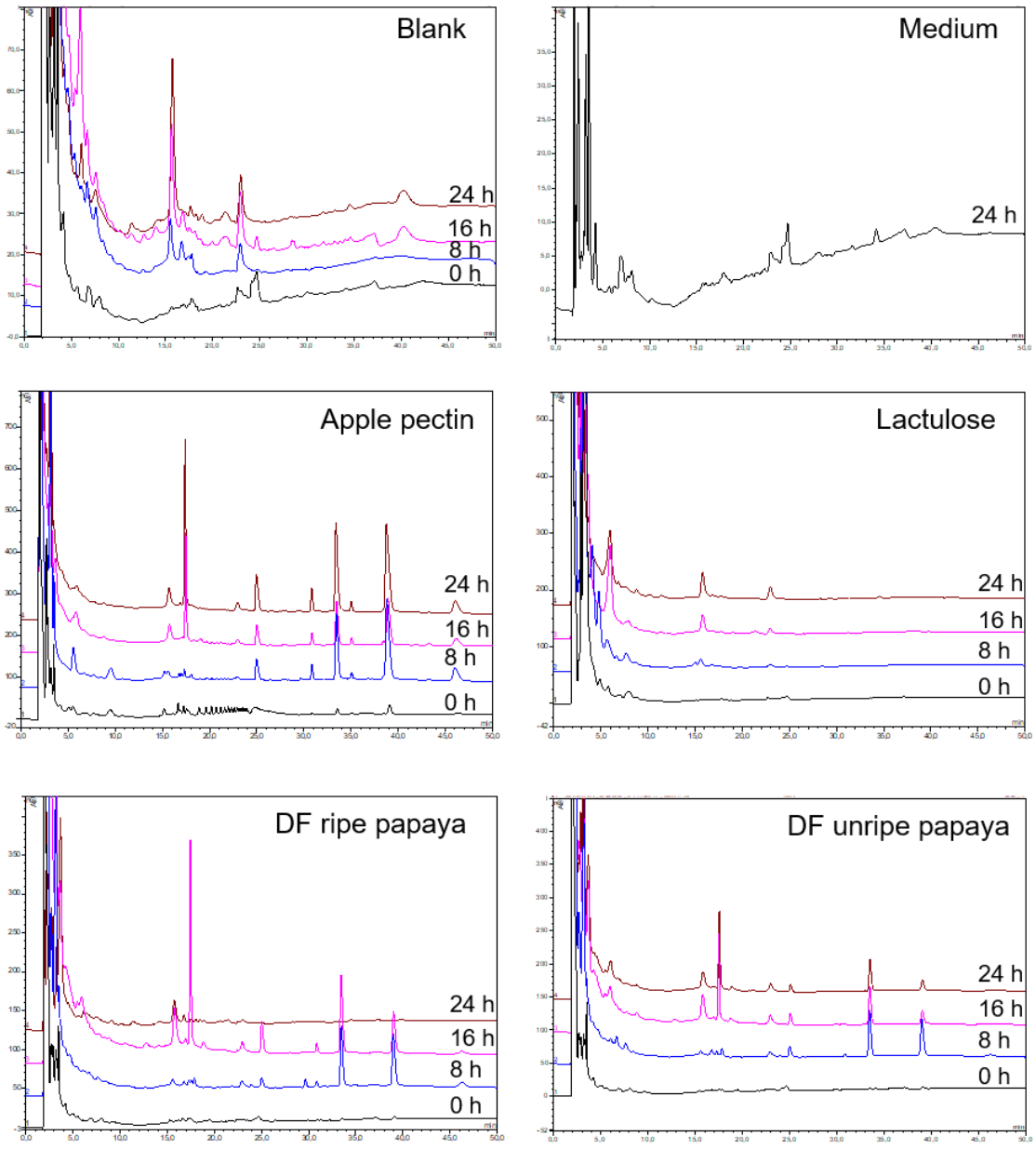

Figure 6. Oligosaccharide of colonic *in vitro* fermentation aliquots.

DF from unripe and ripe papaya had the Weighted UniFrac distances matrix significantly different from the others carbohydrate treatments at 24 h of fermentation, so we decided to evaluate what could be changing in the bacteria profile focusing on DF from unripe and ripe papaya. As lactulose also significantly increased acetate and butyrate production, we focused on the analysis of the blank at 0 h, as well as lactulose and DF from unripe and ripe papaya after 24 h of fermentation. In this way, the differences between these groups can be assigned.

On the operational taxonomic unit (OUT) heatmap we can clearly see changes on the abundance of microorganism sequences and the DF extracted from both unripe and ripe papaya seems to present a distinct bacterial profile compared to the other groups (**Figure 7A**). At the genus level *Clostridium* and *Bacteroides* were the main difference assigned between the fermentation of DF from (unripe and ripe) papaya compared with the blank at 0 h and lactulose at 24 h (**Figure 7B**). *Bacteroides* may produce acetic acid and propionic acid from carbohydrates [39], and *Clostridium* is usually assigned as a producer of butyrate, specifically the *Clostridium XIVa* sp. [41]. This later specie was not identified in the samples, but a considerable increase in *Clostridium* genus was seen after fermentation of DF from papaya, especially for DF from ripe papaya. Furthermore, *Clostridiaceae 02d06* sp. was found increased in the batch incubated with DF from unripe papaya, and this *Clostridiaceae* is usually regarded as a species that uses DF for fermentation and is an indicator of a healthy microbial balance [43,44].

Interestingly, *Prevotella*, which was diminished after fermentation of lactulose and DF from ripe papaya, was still abundant after fermentation of unripe papaya. *Prevotella* (along with *Bacteroides*) is considered a versatile saccharolytic microorganism, being one of the responsible for polysaccharide digestion in the human large intestine [45]. There are others butyrate producers bacteria including *Lachnospiracea incertae sedis* sp., *Faecalibacterium* sp., and *Butyricicoccus* sp. [41]; however, these bacteria were not enhanced after 24 h of fermentation. It is already known that butyrate is not a major end product of *Bifidobacteria* or *Lactobacilli* as believed before, as it appears that *Clostridium* and *Eubacterium* are the main producers of this SCFA [41,46,47].

Furthermore, *Enterococcus* increase during the fermentation of DF from papaya. *Enterococcus* is a commensal bacterial commonly found in the human intestinal tract that has been related with the production of lactic acid, which can be a precursor of SCFA such as acetate [48,49]. *Bulledia* and *Slackia* only increased after the fermentation of DF from unripe papayas. An enrichment of *Bulledia* was reported for a fecal microbiota of growing pigs fed with extrusion of barley and also with a resistant starch-rich diet [50,51]. As far as we known, it is unknown whether *Slackia* have a role in polysaccharide fermentation, but it has been shown that *Slackia* acts transforming secondary bioactive compounds from plants, such as daidzein from soy and trans-resveratrol from grape vine [52,53].

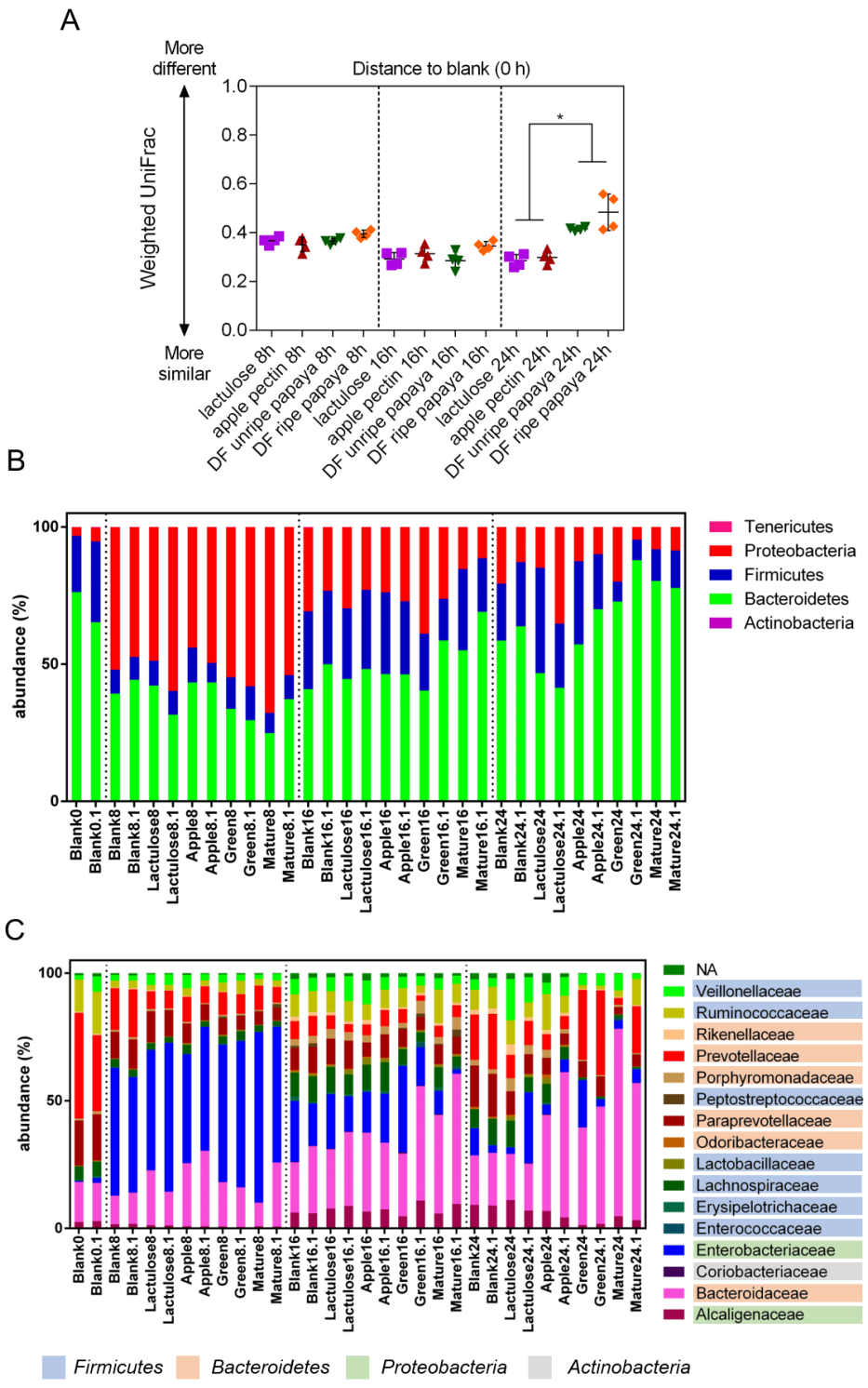

Figure 7. Bacteria profile of different colonic *in vitro* fermentation time points. A) Weighted pairwise UniFrac distances between corresponding to the different carbohydrates used in colonic *in vitro* fermentation. Statistic was calculated: one-way Anova Bonferroni's multiple comparisons test. * $p<0.05$. B) Bacteria percental of abundance at family level.

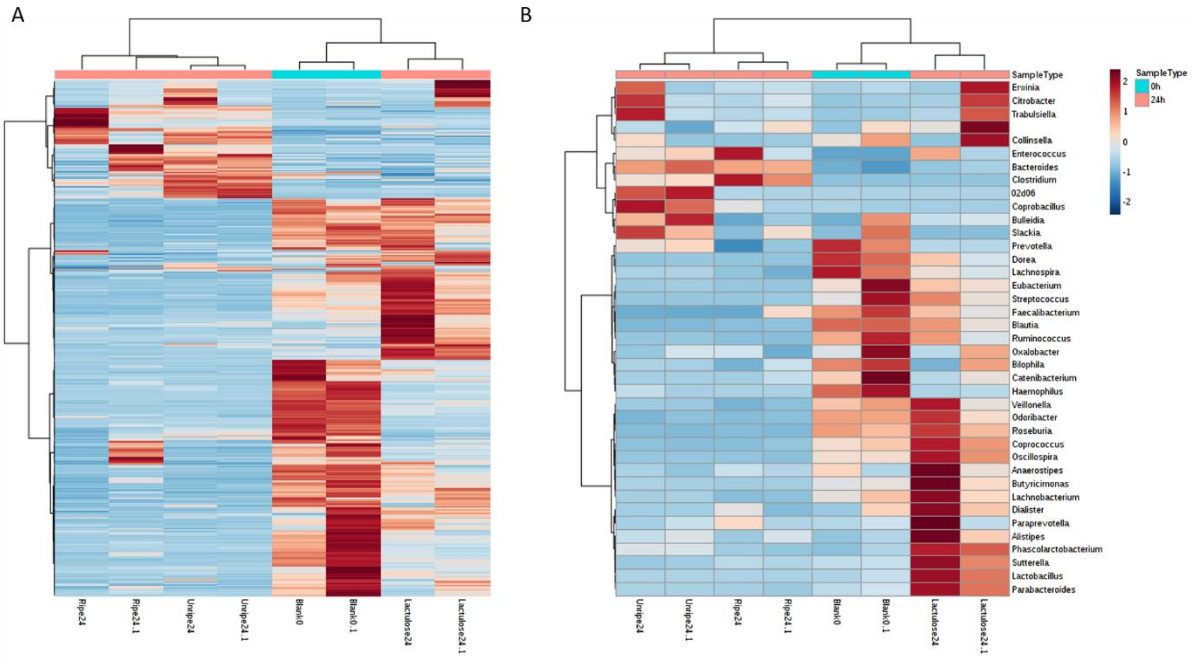

Figure 8. Heatmap of blank 0 h and lactulose, DF from unripe and ripe papayas at 24 h of fermentation. A) OTU heatmap. B) Genus heatmap.

Acetate producing bacteria are *Bifidobacterium* and *Lactobacillus* and also associated with lactulose fermentation [30]. *Lactobacillus* increased for lactulose after 24 h of fermentation; however, no *Bifidobacterium* was found. In another study, no effect on *Lactobacillus* was reported for lactulose fermentation, but for *Bifidobacterium*, *Alistipes*, *Parabacteroides*, *Parasutterella*, and *Anaerostipes* [32]. In our study we also observed after lactulose fermentation an increase in *Alistipes*, *Parabacteroides*, and *Anaerostipes* besides *Lactobacillus*. *Roseburia* was also enhanced for lactulose and it is normally associated with butyrate production, but no pronounced increase in butyrate was observed. This can be explained by a few anaerobes bacteria that can produce not just butyrate but also propionate, such as *Roseburia inulinivorans* and *Coprococcus catus* [5] , thereby explaining why the propionate production was increased and not butyrate.

In summary, besides the similarities between the fermentation profile of lactulose and DF from unripe and ripe papaya, differences in the bacterial profile after fermentation can be easily pointed out. The differences in the structure (and solubility) of DF from unripe and ripe papaya that are dependent of the ripening appears to be the main reasons why the bacteria profile at different time points are different. Even that the increase in SCFA was

seen for DF from both unripe and ripe papaya, the distinct bacterial profile can also influence on the possible health effects.

Conclusions

In this work, the fermentation profile of DF from unripe and ripe papaya were explored in terms of carbohydrate utilization, SCFA production, and effects on the bacterial profile. The DF from ripe papaya seem to be degraded faster than the DF from the unripe papaya, probably by their higher amount of soluble pectin with lower molecular weight of the first. The bacteria profile evaluated by 16s sequencing show similarities as the increase in the abundance of *Clostridium* and *Bacteroides* at genus level. However, DF from unripe papayas appears to induce a specific growth of *Clostridiaceae 02d06*, *Coprabacilus*, *Bulleidia* and *Slackia.* Besides differences on both carbohydrate utilization during fermentation and bacteria profile, the production of SCFA was high after the fermentation of DF from both unripe and ripe papaya compared to other DF, which show that DF from papaya have promising fermentation-related effects.

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Supplementary files

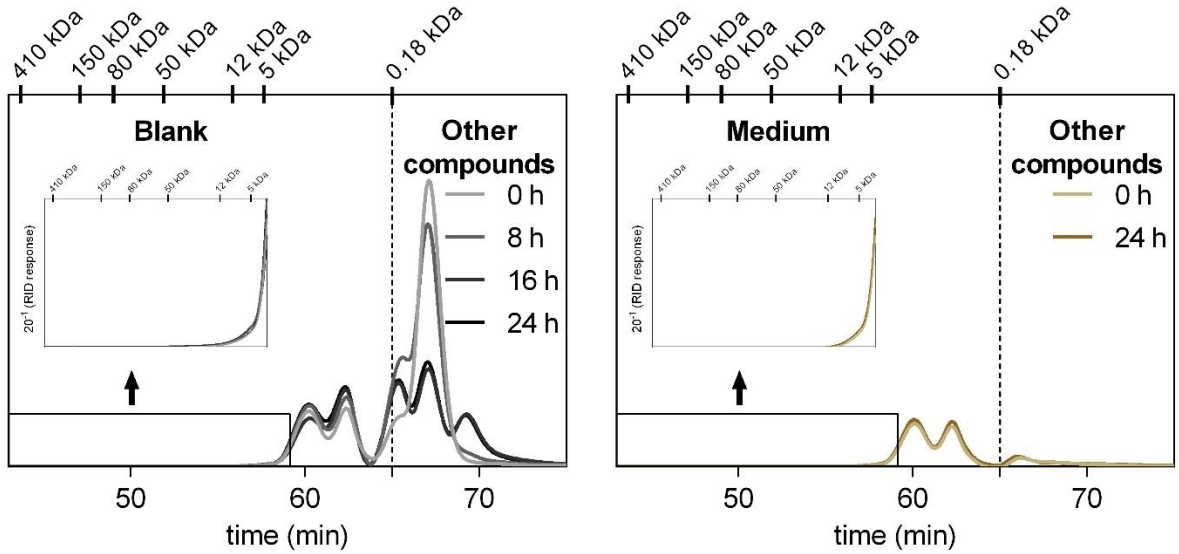

Figure S1. HPSEC from colonic in vitro fermentation: blank and medium treatments.

Chapter 8

Structure-dependent chemopreventive effects of dietary fiber from papaya in rats with colon pre-neoplastic lesions

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Abstract

The consumption of dietary fibers (DF) is for a long time related to beneficial intestinal health habits and with decreasing the risk of colon cancer development. However, DF have a very complex and heterogenous group of molecules, which can differently impact on the decrease in the risk of colon cancer. The naturally modified pectin from papaya fruit has been shown fermentation-related effects and direct effects on colon cancer that are structure-dependent, but until now the *in vivo* results regarding the consumption of DF from papaya is unknown. This study aims to evaluate whether DF from unripe and ripe papaya differently affect the colon lesions development in rats. Chemically-induced animals fed with the DF from ripe papaya showed a significantly decrease in aberrant crypt foci (ACF) formation in the intestine, specially ACF with less than 4 foci. Besides that, the location of ACF inhibition seem to be closer to the proximal colon part than to the distal part. The DF from ripe papayas probably had a different fermentation pattern than the DF from unripe papayas, resulting in distinct interaction with the intestinal cells and microbiota profile. Therefore, the structural differences of DF from papayas – depending on the fruit ripening stage – changes their biological effects, and the ripe DF seem to be more effective as a chemopreventive agent.

Key-words: aberrant crypt foci; azoxymethane; cell wall; colon cancer, dietary fiber; papaya.

Abbreviations: AOM, azoxymethane; ACF, aberrant crypt foci; CRC, colorectal cancer; DF, dietary fibers; DMH, 1,2-dimethylhydrazine; GalA, galacturonic acid; HG, homogalacturonan; IEC, intestinal epithelial cells; Rha, rhamnogalacturonan; SD, standard deviation; TUNEL (Terminal deoxynucleotidyl transferase (TdT) mediated dUTP Nick End Labeling).

Introduction

The consumption of dietary fiber (DF) from plant sources are related to decrease in the incidence of colorectal cancer (CRC) development [1–4]. DF are comprised mainly by carbohydrates that are not hydrolyzed by the gastrointestinal enzymes, but some intestinal bacteria produce the enzymes that can cleave the glycosidic linkages of DF [5]. As result of the fermentation of the DF in the gut, some metabolites are produced including the short chain fatty acids (SCFA) that are associated with the decrease of intestinal pH, as well as with healthy effects such as being energy source for the intestinal epithelial cells (IEC) and promoting benefits that includes the maintenance of the intestinal epithelial barrier integrity [6,7].

The DF from fruits consist mainly of cell wall components including cellulose, hemicellulose, and pectin [8]. Papaya (*C. papaya* L. cv. 'Golden') is a fleshy fruit that usually have a massive pulp softening in just a few days after harvest. The texture of papaya fruit pulp appears to be determined mainly by its cell wall structure, which quickly changes in terms of structure during the ripening. The main fraction of the papaya cell wall that changes after harvest appears to be the pectic fraction, because polygalacturonases and galactanases are the main enzymes that act hydrolyzing the cell wall during papaya ripening [9,10]. Pectin is a complex polysaccharide composed mainly by linear α-(1,4)-linked galacturonic acid (GalA) residues (homogalacturonan, HG) with varying degree of methyl and acetyl esterification [11]. The pectic fraction also consists of branched structures named type I rhamnogalacturonan (RGI), that is the second major pectic fraction of papaya fruit and consists of a backbone of alternate α-(1,4)-GalA and α-(1,2)-rhamnose (Rha) residues [12]. During papaya ripening, polygalacturonases hydrolyze mainly the less soluble pectic fraction, which contains a higher degree of esterification to that of water-soluble pectin. This increase in the activity of polygalacturonases during ripening results in an increased amount of more soluble and more esterified pectin in ripe papaya [10,13,14]. These changes in the DF structure can influence the biological effects of DF and are being pointed out as essential to determine fermentation-related effects that are associated to a decrease risk of colon cancer [15–18].

Among the animal models of chemically induced colorectal cancer models, 1,2 dimethylhydrazine (DMH) and azoxymethane (AOM) are the carcinogens most frequently

used [19]. DMH is an AOM metabolite and both are widely used agents for the induction of colorectal carcinogenesis in rodents [20], which depending on the experimental design can be used to evaluate cancer initiation, promotion and/or metastasis [21]. DMH/AOM-induced colon carcinogenesis may thus be inhibited or enhanced by substances that are administered during the initiation stage. The chemopreventive treatment can begin before exposure to the carcinogen and during the initiation phase, the promotion phase, or through both phases [20]. Aberrant crypt foci (ACF) formation is an early preneoplastic marker of colon tumorigenesis in rats particularly at the promotion stage [22].

The DF from papaya is naturally modified during fruit ripening and different fermentation-related effects and durect effects in colon cancer cells has been related to these structures [13,14]; However, it is unknown whether these DF from unripe and ripe papaya induces *in vivo* effects. In the present study, we incorporate DF from unripe or ripe papaya in the diet of rats and then chemically induce these animals with AOM to develop ACF. In this way, the measurement of the ACF development in animals receiving different diets could indicate a protective effect regarding the inhibition of pre-neoplastic lesions by these diets. Furthermore, as the DF are a food component that should be constantly present in a diet, we continue to incorporate DF from unripe or ripe papaya in the animals diet at both the initiation and the promotion phase after induction with AOM.

Material and methods

Papayas sampling and characterization

Approximately 150 papaya fruits harvested in Bahia (Brazil) were acquired in a commercial fruit market in São Paulo (Brazil), and the ripening were monitored through analysis of internal pulp firmness and peel color according to Fabi et al. (2007) [23]. In addition to the internal pulp firmness, the measurement of the external (whole) pulp was assessed by compression of the fruit with a cylindric probe (4 cm diameter) and analysis using a TA XTplus Texture analyzer (Stable Microsystems). This latter method was used to measure the whole papaya firmness without causing any bruise in the fruit. In this way, we aimed to correlate the external (whole) papaya firmness with the internal (pulp) firmness – this latter is measured on fruit pulp slices. After defining a correlation between the internal pulp firmness and the external fruit firmness it was possible classify the fruits as “unripe”

or “not unripe” without slice the fruits. The fruits classified as “unripe” after measurement of the external fruit firmness were sliced, immediately freeze in liquid nitrogen, and storage in a freezer to further extraction of DF from unripe papaya. Fruits that were determined as “not unripe” were allowed to ripe in a room with controlled temperature (22 ± 0.5 ºC) until they were fully ripe. These ripe fruits were sliced, immediately freeze in liquid nitrogen, and storage in a freezer to further extraction of DF from ripe papaya.

Dietary fiber extraction

Unripe and ripe papaya pulp were freeze-dried and then grinded on a M 20 Universall mill (IKA-Werke GmbH). The powder was further incubated with methanol:chloroform (1:1 v/v) under reflux for 2 h at 70 °C to extract lipids, inactivate enzymes and remove pigments. The solid residue was then washed with 80% boiling ethanol for 1 h under reflux twice for monosaccharide removal and were also washed with three volumes of acetone for drying purposes. Finally, the residue was dried at ambient temperature, resulting in the total cell wall (DF) from papaya.

***In vivo* experiment**

The experiment was done after the approval of the Ethics Committee for Animal Research of the School of Pharmaceutical Sciences at the University of São Paulo (protocol #543). Males Wistar rats with six weeks and weighing 60–80 g were obtained from the colony of the Biomedical Institute at the University of São Paulo. The animals were maintained inside cages containing 4 animals each at a controlled temperature of 22.0 ± 0.5 °C with 12 h light–dark cycle and receiving water and standard commercial AIN-93G diet *ad libitum*. After 1 week of acclimatization, two groups of animals had the dietary fiber of the AIN-93G totally substituted by the DF from unripe or ripe papaya. Four groups were used in the study: Rats not treated with AOM and feed with standard AIN-93G diet (negative control; n = 4), AOM-induced rats feed with standard AIN-93G (AOM control; n =8), and AOM-induced rats feed with AIN-93G but with the DF substituted by DF from unripe or ripe papaya (unripe papaya DF group and ripe papaya DF group; each group with n = 8). Therefore, except for the negative control, all rats received two doses of azoxymethane (AOM; 15 mg/kg body weight; **Figure 1**) on the second week after receiving the diet [24]. After 11 weeks of the first AOM treatment (13 weeks after receiving the diet)

the animals were euthanized and the colon portion – after cecum until the most distal colon part – was resected and washed with sterilized saline solution. The feces and the cecal content were collected and immediately frozen in liquid N_2 and stored at −80 °C. The intestine was longitudinal divided, and half part was immersed in 70% ethanol at 4 ºC. The other half part of the intestine had the mucosa scraped, which was immediately frozen in liquid N_2 and stored at −80 °C.

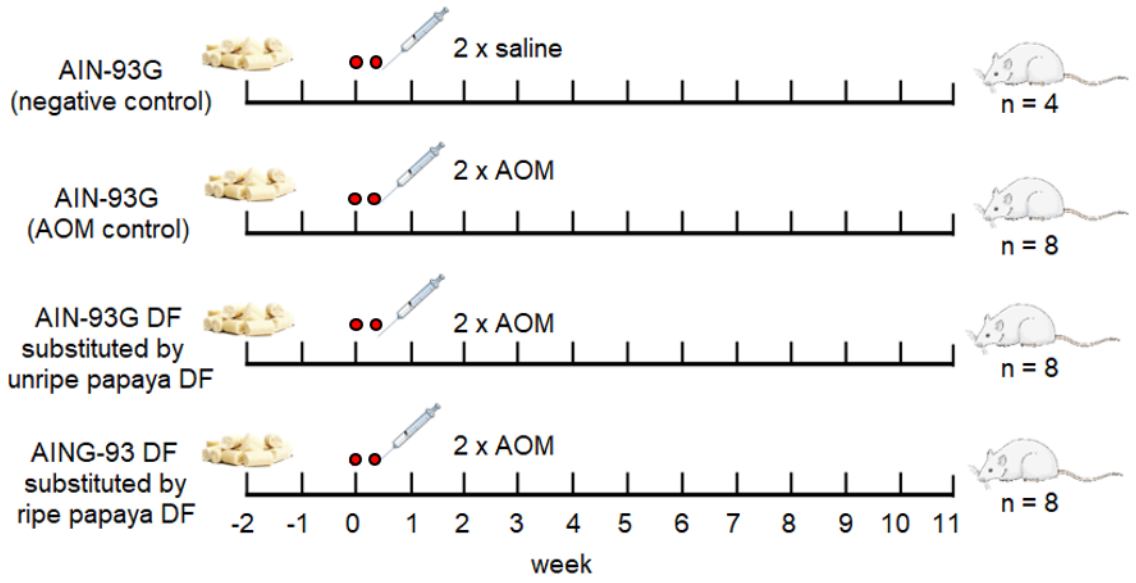

Figure 1. Experimental design of the study.

Aberrant crypt foci (ACF)

The hemicolon segments fixed in 70% ethanol were stained for 5 min with 0.02% methylene blue in phosphate-buffered saline (PBS; 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, pH 7.6). The ACF were analyzed as previously described [25,26] with an inverted microscope (Axiovert 40C, Carl Zeiss Microscopy GmbH, Jena, Germany) at 40× magnification. The total number of ACF and ACF multiplicity in each focus (number of crypts/ACF) was recorded. Data were expressed as ACF/hemicolon segment area (ACF/cm^2).

Statistical analysis

The results were expressed as the mean ± standard deviation (SD). Parametric distribution of data was tested using Shapiro-Wilk normality test. Data were analyzed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). One-way ANOVA with Tukey's (to assess differences between all groups) or Dunnett's or Dunn's multiple comparisons test (to assess differences between the control and two or more groups) were used as post hoc tests. Student's t-test was applied to analyze the differences between two groups. Significance was set at $p < 0.001$***, $p < 0.01$** and $p < 0.05$*.

Results and Discussion

Our group has been intensely studying the structure of DF from unripe and ripe papayas, and one of the challenges regarding this matter is the obtention was how we could obtain the DF from of the unripe papaya, since the fruit ripening occurs very fast. Papaya is harvest unripe (~ ¼ yellow color in the peel) and after 1-2 days the fruit starts the softening process of the pulp from inside to outside because of the increased ethylene production [23], it is unfeasible to go to the field and harvest more than 140 fruits for a study since papaya in Brazil is normally cultivated in the state of Bahia and Espírito Santo – which is at least at 800 km of distance from the laboratory. Therefore, we decided to acquire fruits in São Paulo market where they were used to receive papaya with 1-2 days after harvest after and it was transported refrigerated (~ 15 ^{o}C). The analysis of pulp texture is a destructive method in which the fruit is cut in a half and using a texturometer with a determined probe, it is possible to measure the internal pulp firmness. Since de quantity of papayas were higher than we were used to work in the *in vitro* experiments, we needed a methodology that indicated the ripening without being destructive. Therefore, we decided to determine whether the measurement of the external papaya firmness by pressuring the fruit with a specific larger probe could be correlated to the internal pulp texture thus indicating the onset of ripening. For the unripe papayas, the external firmness above to 40 N was correspondent to the papayas with internal firmness around 20 N, a firmness represented by the "crisp" appearance of the pulp, and thus showing no or little influence by the pectinases. For the ripe papayas, the external firmness between to 30 and 10 N were correspondent to the papayas with internal firmness below 10 N, a firmness represented by

the ideal consumption, since the pulp could be eaten by a spoon but not soften. These values were established as the cut-off for this study (**Figure 2A**). The values of internal texture achieved were in accordance with the literature [9,10,23]. Therefore, with the cut-off determined, the papayas were acquired in batches of a maximum of 25 fruits/day as soon as the market received the stock. For each papaya fruit acquired it was determined the external texture and defined whether the papaya was classified as unripe or whether the papaya was classified as not unripe. Unripe papayas were sliced, and then frozen, and the not unripe papaya were left to ripe until the appropriate cut-off of the external texture for a ripe fruit (**Figure 2B**). At least 25% of the fruits had also their internal firmness measured after slicing, confirming the correlation between the external and internal texture previously determined (not shown). At total, 74 fruits classified as unripe and 74 fruits classified as fully ripe were obtained and used DF extraction. Unripe papayas had 47 kg at total, being 35 kg of pulp, whereas ripe papaya had 41 kg, being 32 kg of pulp. The hue color was also measured (**Figure 2C**). The papayas peels were greener at unripe fruits as expected, and for the riper fruit the peel were more heterogenous, with parts of the fruit varying from yellow to orange color. Besides that, the water-soluble fraction extracted from the DF of unripe papaya showed a yield of 17%, whereas the ripe papaya had a yield of 30%. These results were in agreement with analysis of the water-soluble fraction yield for unripe and ripe papaya obtained from previous studies [13,14,27], thereby confirming that the fruits were appropriately selected according to their ripening stage.

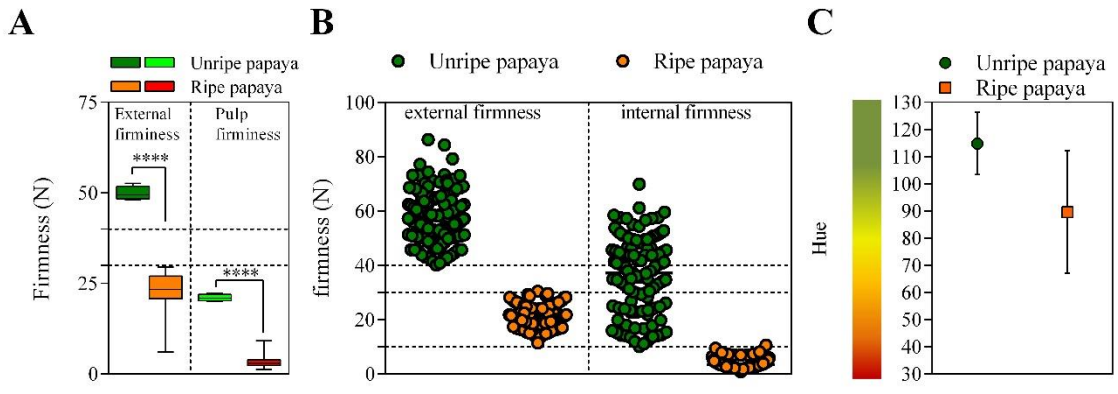

Figure 2. Characterization of papaya ripening. (A) Cut-off definition of external firmness related to unripe and ripe papaya (defined by internal firmness values; n = 29 papayas). (B) External and internal firmness of papaya used as source for DF extraction in the present study. Cut-off definition: external firmness for unripe papaya ≥ 40 N; external

firmness for ripe papaya ≤ 30 N ≥ 10 N. (C) Hue color of papaya used as source for DF extraction in the present study (at least 105 and 187 measurements were performed in unripe and ripe papaya). **** p value < 0.0001 according Student's t-test.

After defining the ripening stage and the papayas sampling, the DF extracted from unripe and ripe papaya and were incorporated to the AIN-93G standard chow by substituting the amount of standard DF (5% w/w of the chow) for DF from unripe or ripe papaya fruit. After two weeks of the maintenance of the standard diet (negative and AOM group) or substitution of the DF by DF from unripe or ripe papaya, the animals were treated with two injection of intraperitonially saline (control) or AOM (15 mg/kg body weight) in a 3-day interval (**Figure 1**). All animals started with an initial weight of 76.69 ± 6.74 g and reach in the final of experiment with a final weight of 365.94 ± 30.22 g (**Figure 3A**). The differences between the initial and final weight among groups were not significantly.

After the second week that the rats were already receiving the chow with or without DF from papaya (weeks –2 and –1), they receive two dosages of saline solution or AOM solution. The rats that received AOM solution tended to maintain or lose weight between the first and second dose, but after three days of the injection they return to increase the weight. The negative control group that only received injection of saline solution had the tendency of gain more weight throughout the experiment compared to the groups that were treated with AOM, because they do not have an impact of receive a carcinogen. Besides that, on the second week, the animals that received AOM solution consumed less chow than the control group and consequently are less prone to gain weight (**Figure 3B**). However, considering the whole experiment duration, the average chow consumption per kg of each animal had no significant difference between the groups. Therefore, the animals weight gain and chow consumption, did not differ between the groups.

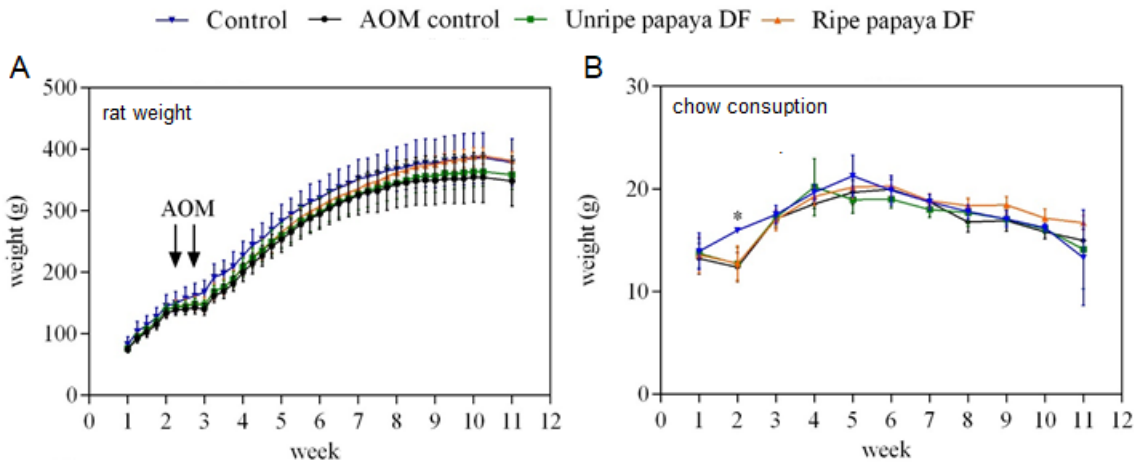

Figure 3. Rat weight and chow weight during the experiment. (A) Rats weight. (B) Avarege chow weight calculated per rat. Data were shown as mean ± SD. *p < 0.05 according to Dunnett's test (all groups compared with the control).

The rat colon was analyzed regarding the ACF quantity, the most proximal part analyzed was the part of the colon next to the cecum, whereas the most distal part of the colon analyzed was the one closer to the rectum. There were significant differences between the total percentage of ACF found in the group that received DF from ripe papaya compared with the AOM control group (**Figure 4A**). The main differences observed was in the more proximal part of the colon and not in the more distal part. Besides that, when we segregated the ACF by their number of foci, we can observe a significant difference for the total ACF counting with less than 4 focus. This difference seems to be mostly associated with the proximal colon part (**Figure 4B**).

In another studies using *in vivo* models with AOM or DMH [20] – different effects regarding DF are seen. Galacto-oligosaccharides had a protective effect against the development of colorectal tumors in rats using AOM model [15]. Slowly fermentable fibers such as wheat bran and oat bran were more protective than rapidly fermentable fibers such as fructooligosaccharides in AOM rat model in reducing the formation of colon tumors [17]. Rats fed with DF source from pectin do not showed a decreased ACF formation [31]; however the pectin source and characterization was not provided, being difficult to establish an structural-dependent effect. Apple pectin reduced the occurrence of colon cancer induced by AOM or DMH [32,33]. Dietary long-chain inulin suppresses AOM-induced ACF formation [34] and even comparing the short-chain inulin with long-chain

inulin, the last showed better effects in inhibits AOM formation [35]. DF from cooked common beans (*P. vulgaris* L., cv Negro 8025) inhibited colon carcinogenesis at an early stage by inducing cell cycle arrest [36,37]. DF of barley reduced the incidence the AOM-induced aberrant crypt foci development [38]. In AOM treated A/J Min/$^{+}$ mice fed with inulin, cellulose or brewers spent grain showed that the type of DF may play a pivotal role in the development of CRC, since just inulin suppressed colonic tumorigenesis [39]. Thus, specific DF structure should be studied in order to establish the specific effects of each DF, especially pectins, since their results in the literature can be very contradictories – probably because of their different structures that is not always characterized.

According to our data, one could speculate that the ripe DF were more active in inhibiting the ACF formation and/or propagation mostly in the proximal part of the intestine. This could be due to the higher quantities of soluble fibers the ripe DF have, since the ripening process transform insoluble to water-soluble pectin, as it has already stated before [10,13,28]. Besides that, the high and low-esterified pectin have different patterns of fermentation in the cecum – being the high-methyl esterified pectin fermented less efficiently than the low-methyl esterified pectin [29,30]. Recently it was shown that rats feed with a high-methyl esterified pectin diet made others DF including arabinoxylans and cellulose shifted the location of fermentation from the cecum to the colon, and this shift not happens when rats were feed with a low-methyl esterified pectin diet [29]. These results from the literature showed differences in pectin structures that affects the fermentation pattern, reinforcing that the papaya pectin modifications during the ripening can affect the pectin structure and the consequent biological effect. Therefore, the biological activity of the ripe DF in the proximal colon could be answered by raising two hypotheses. The first would be the direct effects of the soluble pectins, in which they could interact with IEC, such as activating some toll-like receptors and even inhibiting the galectin-3, thus decreasing the formation and/or propagation of ACF. The other one would be the fermentation process that occurred in a better way in the ripe DF, since there are more soluble DF, and thus facilitating the microbiota fermentation and SCFA production, such as butyrate which has an established anti-cancer effect. The biological samples (feces and cecum content and scrapped mucosa) are stocked in -80 ^{o}C and the paraffined colon sections are stored in order to further studies be done to establish whether these suppositions can be confirmed. The next steps could include molecular biology studies of

scrapped mucosa, histological studies of the colon sections, the microbiota composition of the feces and cecum content besides their DF colonic composition.

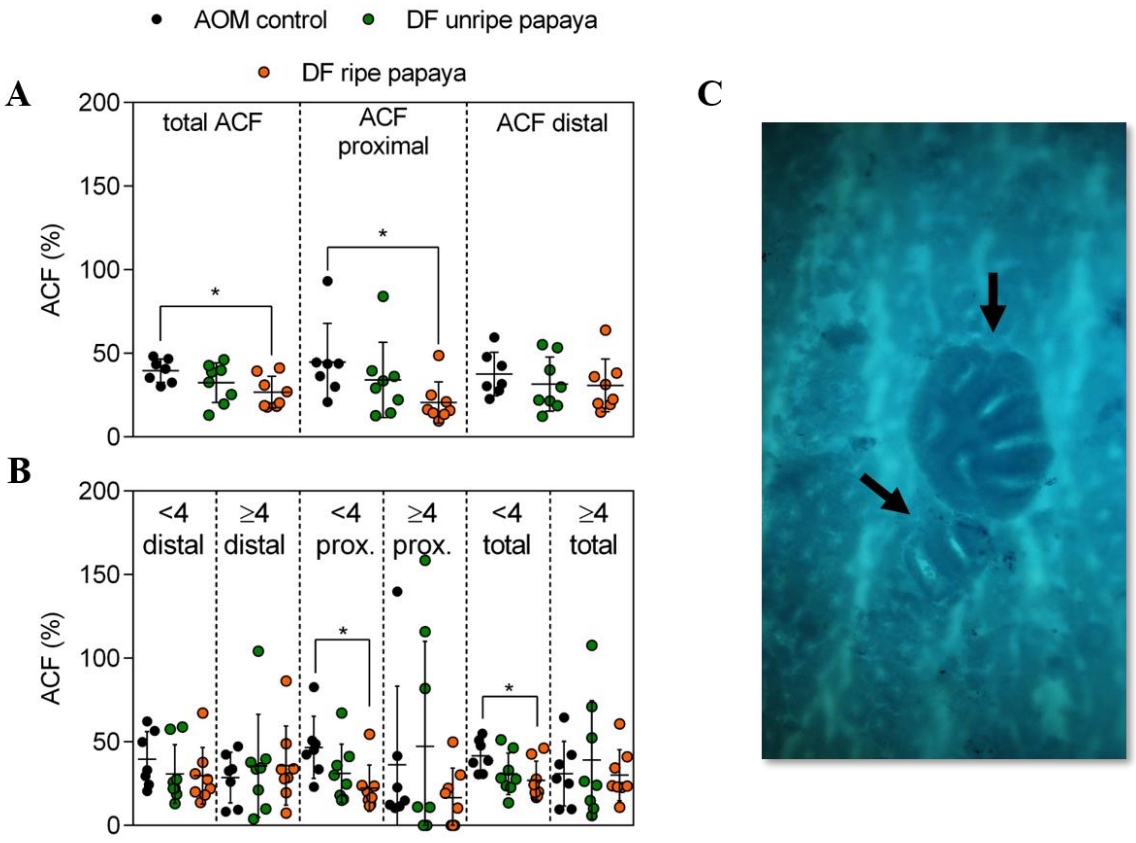

Figure 4. Aberrant crypt foci percentages and representative image. A) Total ACF percentage into more distal or more proximal part of the colon. B) Percentage of ACF with higher or lesser than 4 focus into more distal or more proximal part of the colon. C) Representative of ACF image. The first black arrow indicates an ACF with six focus and the second arrow indicates an ACF with two focus. Data were shown as mean ± SD. Dunn's test, *$p < 0.05$. ACF: aberrant crypt foci.

Conclusions

The papayas ripening challenges regarding the sampling of the unripe papayas were well resolved. We could obtain enough papayas to extract their DF from unripe and ripe fruits and insert theses DF in rats chow. The difference into the animal's diet was just in the DF structure, while all groups received 5% of DF, composed by cellulose (from AIN-

93G), or DF from unripe papaya or DF from ripe papaya. The rats were chemically induced by AOM to develop ACF lesions and the rats fed with ripe papaya DF showed an inhibition of ACF mainly in more proximal colon part. The main change in papayas DF structure is on the pectin structure, since during the ripening the polygalacturonases enzymes are strongly acting and degrading the fruit cell wall [9,10]. The ripe papaya DF may present best results because of the higher quantities of soluble fibers, especially pectins, that could directly act in IEC or be highly fermented by the intestinal microbiota thus producing butyrate. More studies regarding the cecum and colon carbohydrates composition could elucidate whether the utilization of the DF is really happening as hypothesized in this study, together with some molecular biology studies and with the SCFA production and the microbiota composition.

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Concluding remarks and perspectives

Dietary fibers consumption has been related to improving some health effects, as they can increase the stool bulk by gel-forming properties, water holding capacity while having the ability of bind to other organic compounds thus decreasing the cholesterol reabsorption and helping to control the glycemic response. Besides the physical properties, the dietary fibers are also fermented by the intestinal microbiota helping to modulate the bacteria diversity and increasing the beneficial metabolites production, such as short chain fatty acids. The effects of the dietary fibers by the direct interaction with the epithelial intestinal cells or with the immune cells can also occur (**Chapter 3**). However, most of these effects are dependent on the polysaccharide structure with a lack in understanding this structure–function relationship between dietary fibers and the human health effects. In this work, we contribute to increase the knowledge about the fruit dietary fibers, mainly derived from papayas obtained in different ripening time points. Our innovated idea was to try to establish the relationship between the papaya changes in the cell wall during the ripening phenomenon with the structural–dependent effects on cancer cells, on cells pattern recognition receptors, on *in vitro* fermentation, and on *in vivo* experiment. As part of this thesis, we also contributed to the dietary fiber field studying the modified citrus pectin, which was separated into different molecular sizes and their structure–dependent effects on cancer cells were investigated (**Chapter 4-part I)**.

In the **Chapters 1 and 2** we concluded that the fast softening of papaya fruit during ripening is a process caused by a coordinated action of several enzymes in the polysaccharide structure that forms the plant cell wall. The pectinases, mainly endopolygalacturonases and galactanases, were responsible for the mobilization of high molecular size pectins from less soluble to more soluble in water fractions. The solubilized pectins were especially the pectins tightly bound to cellulose/hemicellulose and to each other by calcium bridges.

The dietary fibers from unripe to ripe papayas were extracted and the pectins were directly tested on cancer cells. In **Chapter 4-part II**, we stated that the papaya pectin structure is indeed affected by the coordinated action of several pectinolytic enzymes during fruit ripening. The pectin derived from intermediate papaya ripening time point increased the anti-cancer activity when compared to pectin isolated from other ripening stages. This intermediate papaya ripening time point pectin had smaller homogalacturonans

chains, smaller type I rhamnogalacturonans side-groups, and type II arabinogalactans associated with type I rhamnogalacturonans. The anti-cancer different mechanisms of cell death and migration were dependent on both the dietary fiber structure and the cancer cells mutation. Similar experiments were conducted to the very known pectin – the thermally modified citrus pectin – on **Chapter 4-part I**. The modified citrus pectin fractionated by molecular size resulted in diverse effects on cancer cell proliferation, migration, and aggregation. Those effects were size–, structure–, and cell line–dependent. The smaller sizes showed the strongly anti-cancer effects, and structurally one of these molecules had an enrichment of type I arabinogalactans and another had both fewer branched structures (type I rhamnogalacturonans and type I arabinogalactans) and more de-esterified homogalacturonans oligomers.

The investigation of the papaya pectin interaction with pattern recognition receptors showed in **Chapter 5** suggests that pectin fractions from ripe papayas that have higher degree of esterification and smaller chains, interact and activate all the studied TLR (TLR2, TLR3, TLR4, TLR5 and TLR9). Pectin from unripe papayas that have lower degree of esterification and longer homogalacturonan chains interact with TLR3 and TLR9 in an irreversible way blocking the agonist activation. One more time, it was concluded that the ripening process altered the pectin structures thus altering some biological effects. Besides the water-soluble fraction of the papaya dietary fiber, it was investigated the chelate-soluble fraction, if one could argue during papaya consumption, all fractions of the plant cell wall will be ingested. The chelate fraction is marked for being formed by pectin tightly bound to each other by "egg-boxes" resulted from calcium chelation. In **Chapter 6**, the chelate-soluble fraction obtained from an intermediate papaya ripening time point showed the ability to bind the pro-metastatic protein galectin-3 and to inhibit the colon cancer cell lines proliferation, probably in a galectin-3 dependent and independent way. This chelate-soluble fraction obtained from intermediate papaya ripening had a lower molecular size, and probably the more exposed ramifications could have facilitated the structural interaction with both galectin-3 and cancer cells.

So far, the more soluble dietary fiber from papaya pulp was related to the direct effects to cancer cells, to galectin-3 inhibition and to cells expressing specific pattern recognition receptors. These effects were mainly dependent of the dietary fiber structures and of the papayas ripening stages. The main fraction that presented the best biological

activity was the pectin fraction, and despite the fact the water soluble and the chelate soluble fractions were studied, there might be other fractions that could have some biological effects after being altered by the intestinal microbiota. These fractions include the insoluble pectin (the ones cross-linked to hemicelluloses and to the cellulose microfibrils) and the hemicellulose and cellulose fractions, themselves. The total unripe and ripe papaya dietary fibers were used as substrate for a human colonic *in vitro* fermentation described in **Chapter 7**. The total dietary fibers from ripe papaya was degraded faster than the dietary fibers from the unripe papaya due to the probable easier accessibility of bacteria to higher amounts of soluble polysaccharides with lower molecular weight from the ripe papaya dietary fibers. Besides the differences on carbohydrate utilization by fermentation and bacteria profile, the production of SCFA was the same for both unripe and ripe papaya dietary fibers, but both being higher than lactulose and apple pectin, two standard substances of colonic fermentation. The papaya dietary fibers were also incorporated in AIN-93G chow to evaluate their consumption by rats that were chemically induced to develop pre-neoplastic lesions in the colon (**Chapter 8**). The animals fed with dietary fibers from ripe papayas showed a significantly inhibition in aberrant crypt foci development, mainly localized at the more proximal colon region (near to cecum). Since during the papaya ripening the polygalacturonases enzymes are strongly acting and degrading the fruit cell wall, the main changes in papaya dietary fibers structure is related to pectin solubilization. Thus, the higher quantities of soluble fibers (mainly the pectins) may facilitate the bacteria accessibility increasing the fermentation and could also have some effects acting directly with the intestinal epithelial cells.

Therefore, in this thesis we determined the main changes in papaya cell wall during the ripening, the structural characterization of papaya dietary fibers and the distinct biological effects that are structural–ripening–dependent. During papaya ripening, profound structural changes, mainly in pectin structure, occur leading to different pectin structures that presented differential biological effects. The changes in the structure of the papaya pectin driven by natural ripening provide promising clues regarding the structures of bioactive fruit compounds, especially bioactive polysaccharides that are found in fleshy fruit. Besides that, the results of this thesis could be used as basis to create new and cost-effective approaches to extract and to create pectins with desired structural and biological features.

Further studies are needed to standardize a high-throughput method for the dietary fibers optimal structure isolation, in which structures could have specific target effects in specific groups or for generical beneficial effects for the population. The screening of optimal dietary fibers structure can be done using cell-based assay, using epithelial, immune and transformed cells to study the specific receptor structure interaction. More studies in animals and in humans should be done, in order to be able to identify and pinpoint how the dietary fiber structures influence human heath, by evaluating for example, the gut microbiota, the stimuli of specific immune responses and whether it can improve intestinal barrier function. In this way, it could be possible to select the dietary fiber structures to specific cases allowing a tailored nutrition products development and/or specific food source recommendation. Furthermore, the dietary fiber daily recommendation should be restructured based on different dietary fibers structures, since the effects of its consumption could be completely different.

Appendices

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão de Ética no Uso de Animais - CEUA

CEUA/FCF 44.**2017-P543**

CERTIFICADO

Certificamos que a proposta intitulada **Efeito da pectina de mamões no tratamento de células de câncer colorretal e em modelo in vivo de carcinogênese**, registrada com o nº **543**, sob a responsabilidade do(a) pesquisador(a) **Samira Bernardino Ramos do Prado**, sob orientação do(a) **Prof. Dr. João Paulo Fabi** – que envolve produção ou manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei Federal nº 11.794, de 8 de outubro de 2008, do Decreto Federal nº 6.899, de 15 de julho de 2009, e das normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA) da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (FCF/USP), em reunião de **04 de agosto de 2017**.

Finalidade	Pesquisa Científica
Vigência da autorização	04/08/2017 a 31/12/2019
Espécie/linhagem/raça	*Rato / Wistar*
Número de animais	28
Peso/Idade	100g; 6 semanas
Sexo	Macho
Origem	Biotério - USP

Conforme a legislação vigente, deverá ser apresentado, no encerramento do projeto de pesquisa, o respectivo **relatório final**.

São Paulo, 04 de agosto de 2017.

Profa. Dra. Neuza Mariko Aymoto Hassimotto
Vice-Coordenadora da CEUA/FCF/USP

Av. Prof. Lineu Prestes, 580, Bloco 13 A, Cidade Universitária, CEP 05508-900, São Paulo, SP
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PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Fermentação in vitro das pectinas extraídas de mamões
Pesquisador: JOÃO PAULO FABI
Área Temática:
Versão: 1
CAAE: 43129115.7.0000.0067
Instituição Proponente: Faculdade de Ciências Farmacêuticas da Universidade de São Paulo
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.089.446
Data da Relatoria: 27/04/2015

Apresentação do Projeto:
O surgimento de doenças crônicas não transmissíveis, como o câncer, pode ser influenciado por hábitos pouco saudáveis como uma alimentação com baixo consumo de frutas. A ingestão de fibras alimentares solúveis presentes nas frutas, com destaque para as pectinas, pode contribuir para uma alteração desse panorama. Dentre os vários efeitos benéficos dessa ingestão, atualmente destacam-se a diminuição da incidência do
surgimento e da evolução (metástase) do câncer. Quando as pectinas são metabolizadas pela microbiota intestinal (fermentação colônica), os metabólitos produzidos podem inibir determinados mecanismos de surgimento do câncer. Devido ao fato do mamão papaia ser um fruto carnoso, com grandes quantidades de pectinas solúveis ricas em galactanos, o objetivo do presente trabalho é avaliar os efeitos biológicos das pectinas de mamão extraídas em diferentes tempos de amadurecimento na fermentação colônica in vitro e seus metabólitos tem efeito contra células de câncer. Dessa forma, os resultados obtidos poderão contribuir para o estabelecimento de uma relação entre as transformações bioquímicas das pectinas de mamões decorrentes do amadurecimento e os prováveis efeitos biológicos de sua ingestão, podendo servir de auxílio para o incentivo do consumo dessa fruta ou para o desenvolvimento de alimentos funcionais derivados das pectinas de frutas.

Endereço: Av. Prof. Lineu Prestes, 580, Bloco 13A, sala 112
Bairro: Butantã **CEP:** 05.508-000
UF: SP **Município:** SAO PAULO
Telefone: (11)3091-3622 **Fax:** (11)3031-8986 **E-mail:** cepfcf@usp.br

Objetivo da Pesquisa:

Avaliação dos efeitos biológicos do tratamento de células de câncer colorretal com os metabólitos resultantes da fermentação colônica in vitro das fibras alimentares de mamões verdes e maduros.

Avaliação dos Riscos e Benefícios:

Riscos mínimos, serão fornecidas amostras de fezes, portanto material obtido de forma não invasiva.

Benefícios: Trará maior conhecimento sobre possível ação de componentes de frutas na redução do risco de câncer retal.

Comentários e Considerações sobre a Pesquisa:

A pesquisa pretende demonstrar que alguns componentes presentes em frutos poderiam contribuir para a redução dos riscos de DCNT. Está muito bem elaborada, e certamente os resultados serão muito interessantes. Uma vez comprovada a hipótese poderá trazer ganhos para a população.

Considerações sobre os Termos de apresentação obrigatória:

Adequada.

Recomendações:

Sem recomendações.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Este CEP entende que o projeto pode ser aprovado.

SAO PAULO, 01 de Junho de 2015

Assinado por:
Mauricio Yonamine
(Coordenador)

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