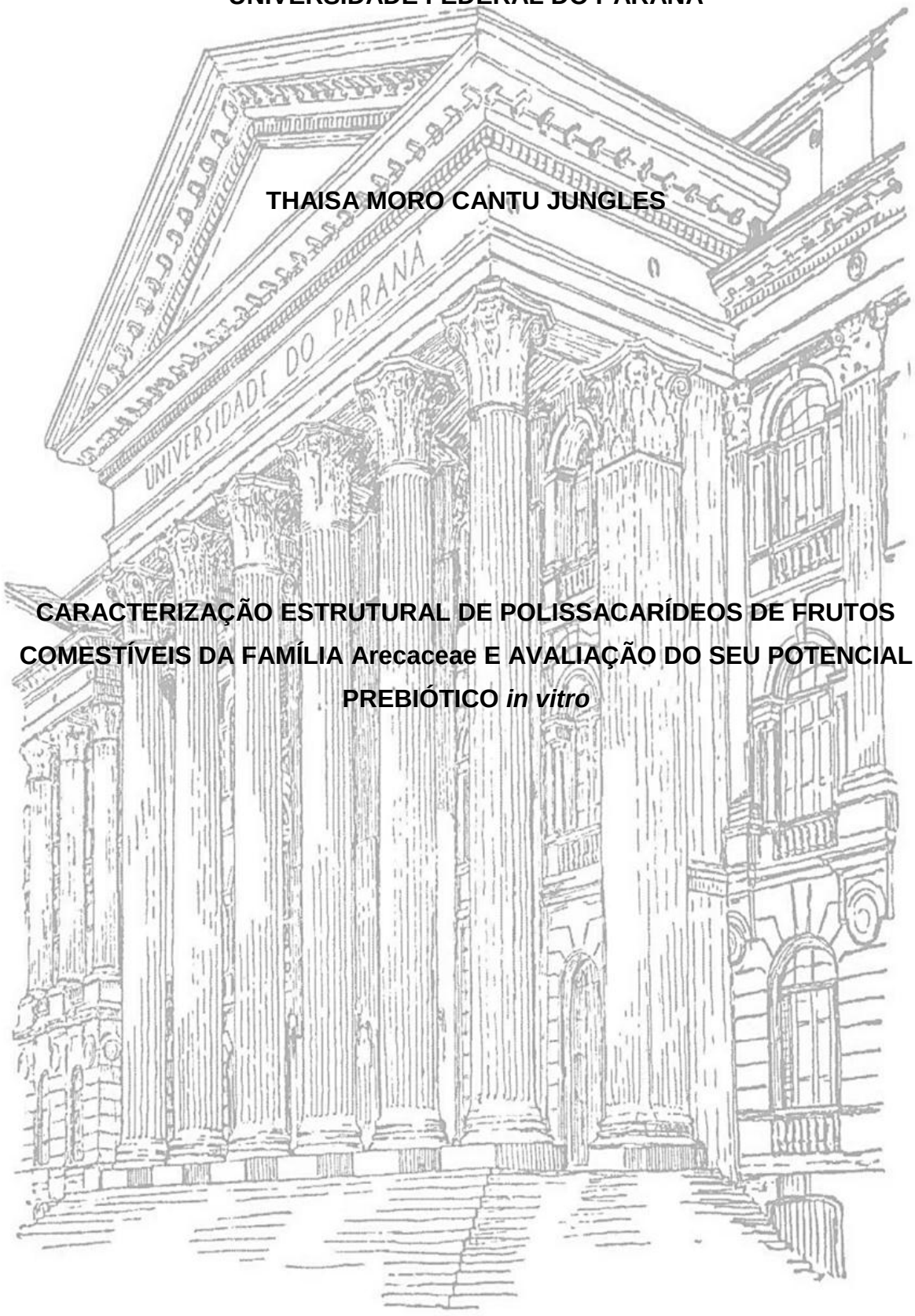
UNIVERSIDADE FEDERAL DO PARANÁ

THAISA MORO CANTU JUNGLES



CARACTERIZAÇÃO ESTRUTURAL DE POLISSACARÍDEOS DE FRUTOS COMESTÍVEIS DA FAMÍLIA Arecaceae E AVALIAÇÃO DO SEU POTENCIAL PREBIÓTICO *in vitro*

CURITIBA
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Tese apresentada ao Programa de Pós-Graduação em Ciências - Bioquímica, do Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Ciências-Bioquímica.

Orientador: Profa. Dra. Lucimara Mach Côrtes Cordeiro
Co-orientadores: Prof. Dr. Thales Ricardo Cipriani; Prof. Dr Bruce Hamaker.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS (BIOQUÍMICA) da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de THAISA MORO CANTU JUNGLES intitulada: CARACTERIZAÇÃO ESTRUTURAL DE POLISSACARÍDEOS DE FRUTOS COMESTÍVEIS DA FAMÍLIA Arecaceae E AVALIAÇÃO DO SEU POTENCIAL PREBIÓTICO in vitro, após terem inquirido a aluna e realizado e realizado a avaliação do trabalho, são de parecer pela sua Aprovação.

CURITIBA, 19 de Dezembro de 2016.

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A Deus, que me concede todas as coisas, dedico este trabalho, meus dias, meus amores e minha vida.

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“Continue a nadar, continue a nadar”

(Dory – Procurando Nemo)

RESUMO

Foi previamente postulado que a parede celular primária de membros da família Arecaceae (plantas monocotiledôneas comelinídeas) possuiria pectinas como principal polissacarídeo não celulósico, ao invés da hemicelulose arabinoxilana que é encontrada nas demais famílias comelinídeas. Entretanto, poucos estudos foram desenvolvidos no sentido de confirmar tal hipótese. Além disso, a estrutura e composição de polissacarídeos de plantas é um fator determinante das funcionalidades que estes poderão exercer quando consumidos pelo homem, como por exemplo, com função atividade prebiótica. O presente trabalho apresenta a caracterização estrutural de polissacarídeos extraídos dos frutos do buriti (*Mauritia flexuosa*), açaí (*Euterpe oleracea*), tucumã (*Astrocaryum aculeatum*) e da pupunha (*Bactris gasipaes*), todos pertencentes à família Arecaceae, com foco nas suas relações filogenéticas, assim como na avaliação do potencial prebiótico *in vitro* de alguns dos seus polissacarídeos. Os polissacarídeos foram extraídos por extrações aquosa e alcalina a quente sequenciais. Foram fracionados por congelamento/degelo, tratamento com solução de Fehling, ultrafiltração, e tratamentos com α-amilase e ácido tricloroacético. As moléculas obtidas foram caracterizadas por técnicas químicas, espectrométricas, espectroscópicas (RMN), cromatográficas (GC-MS e HPSEC) e microscopia eletrônica de varredura (SEM). O potencial prebiótico de alguns dos polissacarídeos obtidos foi avaliado por meio do perfil de fermentação dos mesmos (produção de ácidos graxos de cadeia curta e ramificada, gás e alterações de pH) durante fermentação *in vitro* usando microbiota intestinal humana. Hemiceluloses foram encontradas como principal componente polissacarídico no tucumã (arabinoxilana> arabinana> glucuronoxilana> xiloglucana) e açaí (xilana linear). Este último também apresentou menores porções de polissacarídeos pécticos identificados como arabinogalactana do tipo II e homogalacturonana (DM = 88%). Com relação ao buriti, polissacarídeos pécticos ricos em arabinose foram encontrados em quantidades similares às hemiceluloses. Na pupunha, uma homogalacturonana (DM = 70%) com pequenas porções de xilogalacturonana e ramnogalacturonana do tipo I foram obtidas a partir do extrato aquoso. Entretanto, a caracterização de polissacarídeos da sua fração alcalina não foi realizada. Com relação ao potencial prebiótico dos polissacarídeos obtidos, polímeros com baixa solubilidade como a xilana (extraída do açaí), arabinoxilana e

arabinana (extraídas do tucumã) nativos e tratados com microondas foram submetidos à fermentação *in vitro*. Embora nenhum dos substratos tenha sido fermentado na sua forma nativa, o tratamento com micro-ondas aumentou significativamente a produção de gás e ácidos graxos de cadeia curta, indicando maior fermentabilidade. Em especial, destaca-se a produção de butirato durante a fermentação da xilana tratada com microondas, que atingiu 28% do total de seus ácidos graxos de cadeia curta produzidos em 24 horas de fermentação. Alterações físicas dos polímeros após tratamento com micro-ondas, como teor de solubilidade e estrutura tridimensional foram observadas e podem estar associadas a maior fermentabilidade das frações após tal procedimento. Por fim, pectinas extraídas da pupunha foram submetidos à fermentação *in vitro*, e levaram a mesma produção de ácidos graxos de cadeia curta totais que o controle positivo (FOS), mas com maior produção de acetato (16.2%) e propionato (6.2%) e menor produção de butirato (112%) em relação a FOS. De maneira geral, os polissacarídeos encontrados na parede celular primária da família Arecaceae, apresentam maior variabilidade entre espécies do que previamente se havia suposto. Além disso, a compreensão de como a fermentação de diferentes estruturas químicas leva à produção de metabólitos distintos pela microbiota intestinal humana durante a fermentação de polissacarídeos solúveis e insolúveis aqui realizada, traz novas perspectivas para o desenvolvimento e aplicação de prebióticos.

Palavras-chave: Arecaceae, comelinídeas, polissacarídeos, pectinas, hemiceluloses, atividade prebiótica.

ABSTRACT

It has been previously hypothesized that primary cell walls of Arecaceae family members (commelinid group of monocotyledonous plants) possess pectic polymers as main non-cellulosic constituents, instead of the hemicellulose arabinoxylan which is found in other commelinids families. However, few studies have been undertaken to confirm this hypothesis. Additionaly, the chemical structure of polysaccharides affects the functionalities that they may exert when consumed by man, such as prebiotic activity. This work presents the structural characterization of polysaccharides extracted from the fruits of buriti (*Mauritia flexuosa*), açaí (*Euterpe oleracea*), tucumã (*Astrocaryum aculeatum*) and peach palm (*Bactris gasipaes*), all belonging to the Arecaceae family, focusing on their taxonomic relationships, as well as the assessment of potential prebiotic activities of some of their polysaccharides. Polysaccharides were extracted by sequential hot water and alkali extractions. They were fractionated by freeze/thawing, treatment with Fehling solution, ultrafiltration and treatment with α-amylase and trichloroacetic acid. The molecules obtained were characterized by chemical, spectrometric, spectroscopic (NMR) and chromatographic (GC-MS, and HPSEC) techniques and by scanning electron microscopy (SEM). The prebiotic potential of some polysaccharides obtained was evaluated by their fermentation profile (production of short- and branched-chain fatty acids, gas and pH changes) during an *in vitro* fermentation with human intestinal microbiota. Hemicelluloses were found as the main component in tucumã (galactoarabinoxylan> arabinan> glucuronoxylan> xyloglucan) and açaí (linear xylan). The latter also presented smaller portions of pectic polysaccharides identified as a type II arabinogalactan and homogalacturonan (DM = 88%). Regarding buriti fruits, pectic polysaccharides rich in arabinan were found in similar amounts to the hemicellulosic ones previously found by our research group. In peach palm fruits, a homogalacturonan (DM = 70%) with small portions of xylogalacturonan and type I rhamnogalacturonan were found. However, polysaccharides on its alkali extracts were not evaluated. To evaluate the prebiotic potential of some extracted polymers, low solubility polysaccharides such as xylan (extracted from acai), arabinoxylan and arabinan (extracted from tucumã), native and microwaved, were sumbmitted to an *in vitro* fermentation. Although none of the substrates were fermented on their native forms, the treatment with microwaves significantly increased gas and short chain fatty

acids production, indicating higher fermentability than native polymers. In particular, microwave treated xylan lead to higher butyrate production, which reached 28% of its total short chain fatty acid produced in 24 hours of fermentation. Polymers physical changes after microwave treatment such as solubility and tridimensional structure were observed and may be associated with its increased fermentation profile after this proceadure. Finally, pectic polysaccharides extracted from pupunha were also subjected to *in vitro* fermentation and led to the production of similar amounts of total short chain fatty acids than the positive control (FOS), but with a higher acetate and propionate production (16.2% and 6.2%, respectively) and lower butyrate production (112 %) than FOS. Overall, polysaccharides found in the primary cell walls of Arecaceae family members presented more variations between species than previously hypothesized. Furthermore, understanding how the polysaccharides chemical structures lead to the production of distinct metabolites generated during soluble and insoluble polysaccharides human microbiota fermentation brings new perspectives for the development and application of prebiotics.

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Keywords: Arecaceae, commelinid, polysaccharides, pectins, hemicelluloses, prebiotic activity.

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LISTA DE ABREVEATURAS, SIGLAS E SÍMBOLOS

^{13}C-RMN	Ressonância magnética nuclear de carbono treze
^{1}H-RMN	Ressonância magnética nuclear de hidrogênio
AGCC/ SCFA	Ácidos graxos de cadeia curta
AGCR/ BCFA	Ácidos graxos de cadeia ramificada
AG-I	Arabinogalactana tipo I
AG-II	Arabinogalactana tipo II
Ara*f*	Arabinose furanosídica
Ara*p*	Arabinose piranosídica
AX	Arabinoxilana
$BaCO_3$	Carbonato de bário
D_2O	Água deuterada
Da	Daltons
DEPT	*Distortionless Enhancement by Polarization Transfer*
DF	Fibras dietéticas
DM / DE	Grau de metil-esterificação
DMSO	Dimetilsulfóxido
DMSO-d_6	Dimetilsulfóxido deuterado
FOS	Frutooligossacarídeos
Fuc*p*	Fucose piranosídica
Gal*p*	Galactose piranosídica
Gal*p*A	Ácido galacturônico
GAX	Glucuronoarabinoxilana
GC-MS	Cromatografia gasosa acoplada à espectrometria de massa
Glc*p*	Glucose piranosídica
Glc*p*A	Ácido glucurônico
GPC	Cromatografia de gel permeação
H_2SO_4	Ácido sulfúrico
HG	Homogalacturonana
HM	Pectinas com grau de esterificação superior a 50%
HPSEC	Cromatografia de Exclusão Estérica

HSQC	*Heteronuclear Single Quantum Coherence*
kDa	Kilodaltons
MHz	Megahertz
MW	Microondas
M_w	Massa molecular
$NaBD_4$	Borodeuterito de sódio
$NaBH_4$	Borohidreto de sódio
NaN_3	Azida de sódio
$NaNO_2$	Nitrito de sódio
NaOH	Hidróxido de sódio
PGA	Ácido poligalacturônico
RG-I	Ramnogalacturonana tipo I
RG-II	Ramnogalacturonana tipo II
Rha*p*	Ramnose piranosídica
RI	Índice de refração
RMN / NMR	Ressonância magnética nuclear
SEM	Microscopia eletrônica de varredura
TCA	Ácido tricloroacético
TFA	Ácido trifluoacético
UA / AU	Ácido Urônico
XG	Xiloglucana
XOS	Xilooligossacarídeos
Xyl*p*	Xilose piranosídica
δ	Deslocamento químico expresso em ppm

SUMÁRIO

1. INTRODUÇÃO

A Floresta Amazônica é considerada o maior reservatório natural da diversidade vegetal do planeta, sendo detentora de alta diversidade de espécies frutíferas ainda pouco estudadas (OLIVEIRA; AMARAL, 2004). Fazem parte dessa diversidade, um grupo muito particular de plantas pertencentes à família Arecaceae, popularmente conhecidas como “palmeiras” (MIRANDA, 2001). Dentre os frutos comestíveis da família Arecaceae encontrados na Amazônia destacam-se o buriti (*Mauritia flexuosa*), o açaí (*Euterpe oleracea*), o tucumã (*Astrocaryum aculeatum*) e a pupunha (*Bactris gasipaes*).

Taxonomicamente, a família Arecaceae, encontra-se como um grupo entre monocotiledôneas comelinídeas, ou seja, monocotiledôneas que possuem ácido ferúlico na sua parede celular (HARRIS et al., 1997). A maior parte das plantas comelinídeas possui a hemicelulose glucuronoarabinoxilana como principal polissacarídeo não celulósico da parede celular primária. Entretanto, para membros da família Arecaceae, foi postulado que possuiria uma composição de polissacarídeos similar às plantas não comelinídeas e dicotiledôneas, ou seja, ricos em pectinas com pequenas quantidades de polímeros hemicelulósicos (HARRIS, 2005; HARRIS et al., 1997). Notavelmente, tal hipótese foi gerada com base em análises de poucas espécies da família Arecaceae (CARNACHAN; HARRIS, 2000; HARRIS et al., 1997).

Polissacarídeos da parede vegetal podem exercer uma variedade de efeitos biológicos na saúde humana dependendo de sua estrutura química. Entre eles, polissacarídeos prebióticos escapam à digestão no trato gastrointestinal superior e podem ser fermentados por bactérias do intestino grosso, levando à alterações na composição e/ou atividade de bactérias do intestino (GIBSON et al., 2004; GLENN; ROBERFROID, 1995). Entre os metabólitos produzidos durante a hidrólise de polissacarídeos por estas bactérias estão os ácidos graxos de cadeia curta (AGCC) que possuem uma gama de efeitos biológicos conhecidos como o controle de infeção por patógenos, trofismo de células colônicas, regulação do metabolismo lipídico, entre outros (RÍOS-COVIÁN et al., 2016; WONG et al., 2006). Propriedades físicas de um polímero (como viscosidade, solubilidade, tamanho, tipos de monossacarídeos e ligações químicas envolvidas, etc.) pode influenciar sua

suceptibilidade à fermentação por bactérias do intestino humano, bem como o perfil de metabóltios produzidos neste processo (HAMAKER; TUNCIL, 2014).

Visto que a composição de polissacarídeos que compõe a parede celular primária de membros da família Arecaceae é pouco investigada na literatura, a avaliação da estrutura química de polissacarídeos do buriti, tucumã, açaí e pupunha pode ser usada como ferramenta para uma maior compreensão de suas relações estruturais pelo ponto de vista taxonômico. Além disso, uma vez que polissacarídeos podem atuar biologicamente sobre a microbiota intestinal humana, produzindo efeitos benéficos para a saúde do homem e que aspectos estruturais de polissacarídeos estão relacionados às suas propriedades prebióticas, justifica-se a avaliação do perfil de fermentação de polissacarídeos dos frutos da família Arecaceae pela microbiota intestinal humana.

2. REVISÃO BIBLIOGRÁFICA

2.1 A PAREDE CELULAR VEGETAL

As paredes celulares das plantas vasculares são responsáveis por grande parte do carbono fixado durante a fotossíntese e correspondem a maior parte de sua biomassa (HARRIS, 2005). Todas as paredes de plantas vasculares possuem uma construção semelhante, que consiste em dois domínios: um domínio fibrilar formado por microfibrilas de celulose situadas em um domínio matriz, com elevadas proporções de polissacarídeos não celulósicos que variam estruturalmente, juntamente com proteínas estruturais, glicoproteínas e compostos fenólicos que também podem estar presentes na matriz da parede (BACIC; HARRIS; STONE, 1988; CARPITA; MCCANN, 2000; HARRIS, 2005).

Entretanto, polissacarídeos da parede celular podem apresentar extensa variação não somente entre diferentes grupos taxonômicos, mas também entre diferentes tecidos de uma mesma planta. Nos vegetais superiores, três tipos de parede celular são reconhecidos: lamela média, parede celular primária e parede celular secundária (COIMBRA et al., 2004). Dependendo do tecido vegetal analisado e seu estágio de crescimento, um ou mais tipos de parede celular podem ser encontrados. Quando uma célula cresce, as ligações entre os polissacarídeos da parede são rompidas, a célula se expande e novos polissacarídeos são sintetizados e inseridos entre os já existentes. Este processo envolve o rompimento e a formação de numerosas ligações covalentes e não covalentes. Desse modo, as células podem aumentar muitas vezes seu comprimento sem ocasionar enfraquecimento da parede celular (CARPITA; MCCANN, 2000; RAVEN; EVERT; EICHHORN, 2001).

A lamela média é a primeira camada a ser formada durante a divisão da célula. É constituída por uma camada intercelular amorfa de pequena espessura rica em pectinas, que une as paredes primárias de células vizinhas (RAVEN et al., 2001). A parede primária forma-se após a lamela média e é constituída essencialmente por microfibrilas de celulose envolvidas por uma matriz de polissacarídeos

hemicelulósicos e pécticos, apresentando importância na expansão celular (CARPITA; GIBEAUT, 1993). A parede celular secundária forma-se após cessar a divisão celular, internamente à parede celular primária e pode tornar-se bastante espessa devido à deposição de celulose, hemicelulose e lignina, resultando na diferenciação terminal da célula por conferir resistência à compressão e rigidez à parede (CARPITA; GIBEAUT, 1993).

2.2 Relações filogenéticas de plantas e seus polissacarídeos da parede celular primária

Variações na composição de polissacarídeos da parede celular primária podem ser observadas entre diferentes grupos taxonômicos. Carpita e Gibeaut (1993) propuseram uma divisão de paredes celulares de plantas angiospermas em dois tipos (I e II), que variam conforme o arranjo e proporção dos componentes da parede celular. Paredes celulares do tipo I (Figura 1) são representantivas de todas as dicotiledôneas e algumas monocotiledôneas. Por outro lado, paredes celulares do tipo II (Figura 2) são representativas da família Poaceae e algumas outras monocotiledôneas de famílias próximas.

De maneira geral, a parede celular do tipo I (Figura 1) consiste em uma rede de microfibrilas de celulose interligada por xiloglucanas. Outros polímeros como gluco- e galacto-mananas, β-D-glucanas (1→3) e (1→4)-ligadas e glucuronoarabinoxilanas também podem estar presentes interligando microfibrilas de celulose em algumas paredes do tipo I, mas são encontradas em menores proporções do que xiloglucanas (CARPITA; MCCANN, 2000; CARPITA; GIBEAUT, 1993; COSGROVE, 2005; POWELL et al., 1982).

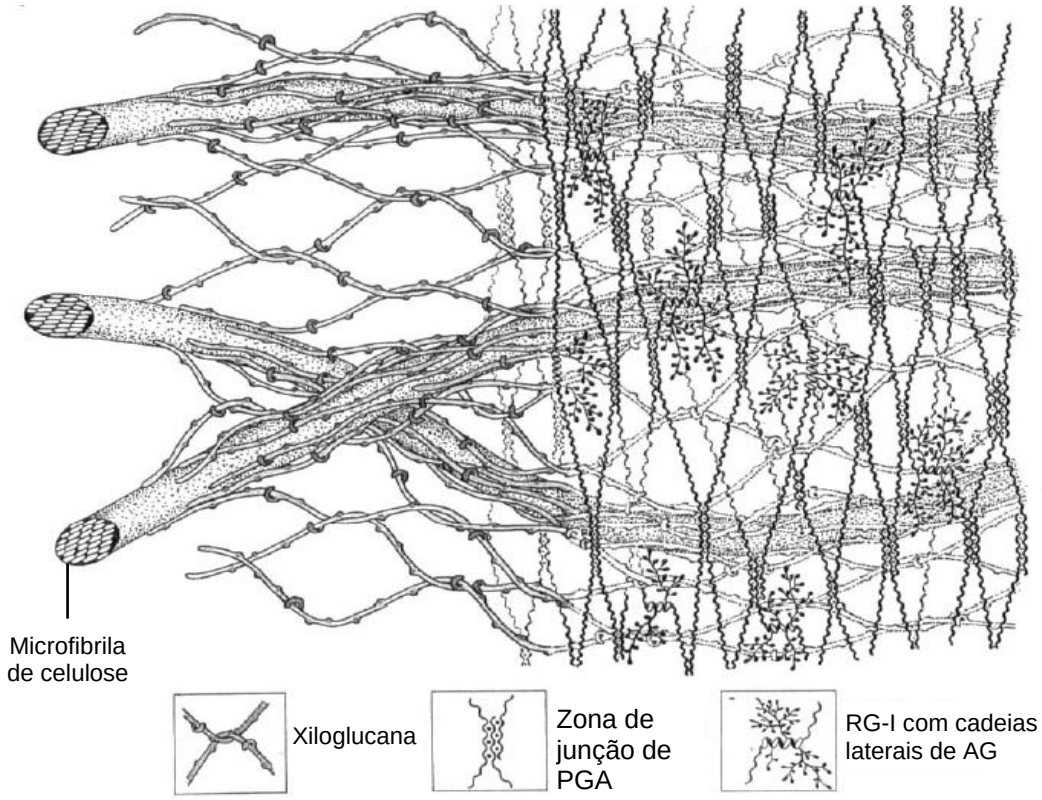


FIGURA 1. ESQUEMA DE PAREDE CELULAR PRIMÁRIA DO TIPO I

FONTE: Carpita e Gibeaut (1993). Abreviações: PGA – ácido poligalacturônico; RG-I – ramnogalacturonana do tipo I; AG – arabinogalactana.

O complexo celulose-xiloglucana corresponde a cerca de 50% da massa da parede e encontra-se embebido em uma matriz formada por polissacarídeos pécticos (~ 30%) que se apresentam na forma helicoidal. Porções de ácido poligalacturônico (PGA) podem se condensar e formar ligações cruzadas com íons de cálcio, formando as “zonas de junção” que ligam duas cadeias de pectinas antiparalelas. As proteínas estruturais correspondem ao terceiro e último componente da parede celular primária, em especial proteínas extensinas que formam “pontes” com outras proteínas, sem interagir, nescessariamente, com componentes polissadarídicos (CARPITA; MCCANN, 2000; CARPITA; GIBEAUT, 1993; COSGROVE, 2005; POWELL et al., 1982).

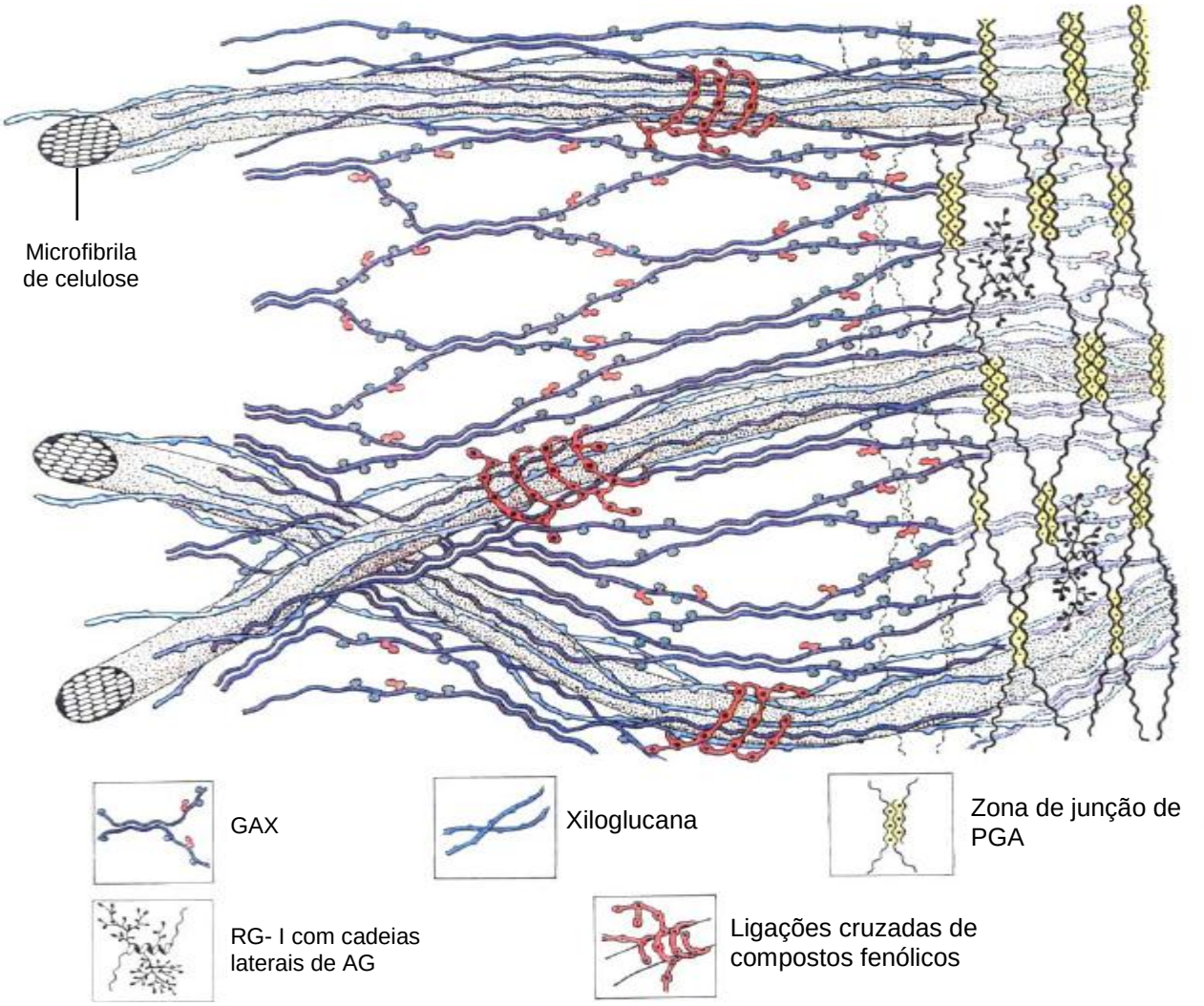


FIGURA 2. ESQUEMA DE PAREDE CELULAR PRIMÁRIA DO TIPO II

FONTE: Carpita e Gibeaut (1993). Abreviações: GAX – galactoarabinoxilana; PGA – ácido poligalacturônico; RG-I – ramnogalacturonana do tipo I; AG – arabinogalactana.

Paredes celulares do tipo II (Figura 2) também possuem microfibrilas de cellulose, entretanto, glucuronoarabinoxilanas (GAXs) encontram-se interligando suas estruturas e compõe a maior parte de seus polissacarídeos não celulósicos e xiloglucanas estão presentes em pequena quantidade. Grande parte da GAX se encontra ligada à parede por meio de compostos fenólicos éter-ligados. Pequenas quantidades de xiloglucanas também podem ser encontradas ligadas às microfibrilas de celulose. Da mesma forma, polissacarídeos pécticos foram identificados em paredes celulares do tipo II em pequena proporção (cerca de 10%). Na fase de

expansão celular, glucanas (1→3) e (1→4), também ocorrem em grande quantidade em paredes celulares do tipo II. A quantidade de proteínas estruturais é menor na parede celular primária do tipo II, entretanto, estas apresentam compostos fenólicos que participam na formação de ligações cruzadas entre os polissacarídeos (CARPITA; MCCANN, 2000; CARPITA; GIBEAUT, 1993).

Relações peculiares da estrutura da parede celular primária de acordo com grupos/famílias de plantas também foram descritas, especialmente para monocotiledôneas, propondo associações táxon-específicas (HARRIS et al., 1997; HARRIS e HARTLEY, 1980). Estudos nesse sentido têm sido impulsionados principalmente devido ao enorme impacto que o sequenciamento genético teve na compreensão da filogenia de plantas, resultando inclusive em uma nova classificação para angiospermas (The Angiosperm Phylogeny Group, 1998; 2003). A descrição precisa de relações taxonômicas proporcionou bases para investigar se a variação em um componente de parede nomeadamente descoberto em um táxon também está presente filogeneticamente em táxons relacionados (HARRIS, 2005).

De maneira geral, as estruturas da parede celular primária relatada para diversas famílias de eudicotiledoneas/dicotiledôneas são semelhantes entre si e similares ao proposto por Carpita e Gibeaut (1993) para paredes celulares primárias do tipo I. Podem ser encontradas grandes proporções de polissacarídeos pécticos, menores proporções de xiloglucanas, e pequena quantidade de heteroxilanas, glucomananas e/ou galactoglucomananas (HARRIS, 2005).

Por outro lado, em monocotiledôneas, maiores variações ocorrem dependendo do grupo e/ou família à qual uma determinada planta pertence (HARRIS, 2005). Com base na presença ou não de ácido ferúlico ligado a polissacarídeos da perede cellular, Harris e Hartley (1980) classificaram monocotiledôneas em dois grupos: comelinoides e não-comelinoides (atualmente conhecidos como comelinídeas e não-comelinídeas) (The Angiosperm Phylogeny Group, 2003). Plantas que não apresentam ácido ferúlico em sua parede celular (não-comelinídeas) incluem as famílias Amarytidaceae, Liliaceae, Dioscoreaceae, Potamogetonaceae e Arecae (Figura 3) (BREMER et al., 2009; GIVNISH et al., 2010).

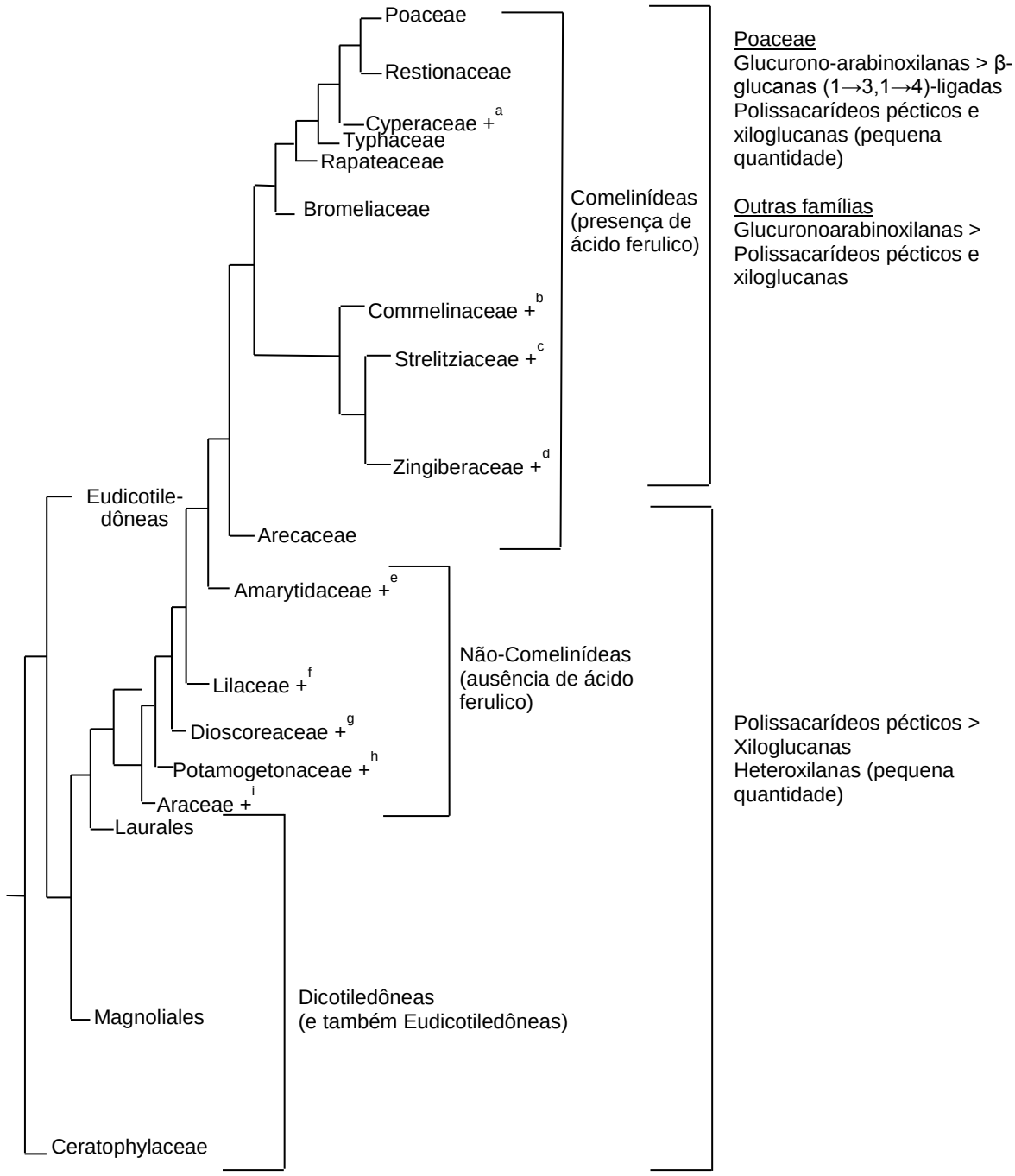


FIGURA 3. CLADOGRAMA INDICANDO RELAÇÕES FILOGENÉTICAS DE DICOTILEDÔNEAS E MONOCOTILEDONEAS COMELINÍDEAS E NÃO COMELINÍDEAS.

Famílias adicionais às apresentadas estão indicadas por +. [a] = Juncaceae e Flagellariaceae; [b] = Pontederiaceae, Philydraceae e Haemodoraceae; [c] = Marantaceae; [d] = Costaceae, Musaceae, Lowiaceae e Heliconiaceae; [e] = Xanthorrhoeaceae, Asphodelaceae, Dracaenaceae, Ruscaceae, Nolinacaceae, Hyacinthaceae, Anthericaceae, Iridaceae, Tecophilaeaceae, Hypoxidaceae e Orchidaceae; [f] = Smilacaceae, Alstroemeriaceae, Colchicaceae, Melanthiaceae (um gênero), Velloziaceae, Pandanaceae e Cyclanthaceae; [g] = Taccaceae; [h] = Melanthiaceae (um segundo gênero), Burmanniaceae e Alismataceae; [i] = Metanthiaceae (um terceiro gênero).

FONTE: adaptado de Harris et al. (1997)

Em contraste, plantas com polissacarídeos da parede celular ligadas ao ácido ferúlico (comelinídeas), incluem as famílias Poaceae, Restionaceae, Cyperaceae, Typhaceae, Rapateaceae, Bromeliaceae, Commelinaceae, Strelitziaceae, Zingiberaceae e Arecaceae (Figura 3) (CHASE et al., 2006; GIVNISH et al., 2010).

A parede cellular primária de não comelinídeas possui grandes proporções de polissacarídeos pécticos e menores concentrações de hemiceluloses (xiloglucanas > heteroxilanas), similar ao que é encontrado nas plantas dicotiledôneas (Figura 3) (HARRIS et al., 1997).

Entretanto, nas plantas comelinídeas, variações entre paredes celulares primárias de plantas de diferentes famílias podem ocorrer. De maneira geral, todas as comelinídeas, com exceção da ordem Arecales, possuem altas proporções da hemicelulose glucuronoarabinoxilana em detrimento de polímeros pécticos e xiloglucanas (Figura 3), similar ao proposto por Carpita e Gibeaut (1993) para a parede celular primária do tipo II. A família Poaceae (gramíneas), que encontra-se situada em posição terminal na árvore filogenética, também apresenta β-glucanas (1→3,1→4)-ligadas em grandes proporções (Figura 3) (HARRIS, 2005; HARRIS et al., 1997).

2.2.1 Família Arecaceae

A família Arecaceae, anteriormente conhecida como Palmae ou Palmaceae, é a única família botânica da ordem Arecales. A família Arecaceae engloba cerca de 3000 espécies pertencentes a 189 gêneros (UHL; DRANSFIELD, 1999). Filogeneticamente, encontra-se como um grupo basal pertencente ao grupo de monocotiledôneas comelinídeas (Figura 3). A respeito de sua parede celular primária, foi previamente hipotetizado que possuiria características semelhantes às plantas não comelinídeas e dicotiledôneas, ou seja, ricas em pectinas com pequenas quantidades de polímeros hemicelulósicos (Figura 3) (HARRIS, 2005; HARRIS et al., 1997). Entretanto, tal hipótese foi gerada com base apenas em análises de composição monossacarídica da parede celular primária de *Phoenix*

reclinata (HARRIS et al., 1997) e composição monossacarídica juntamente com análises de metilação de *Phoenix canariensis* e *Rhopalostylis sapida* (CARNACHAN; HARRIS, 2000).

Mais recentemente, a presença de glucanas foi descrita em frações polissacarídicas da parede celular primária do babaçu (*Orbignya phalerata*) (SILVA; PARENTE, 2001) e tâmaras (*Phoenix dactylifera*) (ISHURD; KENNEDY, 2005). Além disso, polissacarídes ricos em galactose, glucose e manose foram isolados a partir da macaúba (*Acromia aculeata*) (SILVA; MEDEIROS SILVA; PARENTE, 2009), jerivá (*Arecastrum romanzoffiana*) (SILVA; PARENTE, 2010a) e guariroba (*Syagrus oleracea*) (SILVA; PARENTE, 2010b). Entretanto, a proporção desses polímeros em relação ao total de polissacarídeos da parede não foi descrita, dificultando a concepção de associações sobre a parede celular primária pelo ponto de vista taxonômico. No nosso grupo de pesquisa, a presença de α-L-arabinana (1→5)-ligada, β-D-xilana (1→4)-ligada e α-D-glucana (1→3,1→4)-ligada foram observadas nos frutos do buriti (*Mauritia flexuosa*). Estes polissacarídeos representam cerca de metade do total dos polímeros extraídos com água e solvente alcalino, e, portanto, a elucidação completa de seus polissacarídeos da parede celular ainda precisa ser averiguada (CORDEIRO; ALMEIDA; IACOMINI, 2015).

2.3 ESTRUTURA QUÍMICA DE POLISSACARÍDEOS DA PAREDE CELULAR PRIMÁRIA

A estrutura química dos principais polissacarídeos não celulósicos da parede celular primária de plantas (pectinas e hemiceluloses) será brevemente abordada a seguir.

2.3.1 Polissacarídeos Pécticos

Os polissacarídeos pécticos formam o grupo mais complexo de compostos constituintes da parede celular primária de plantas (CARPITA; GIBEAUT, 1993). Existem quatro tipos de polissacarídeos pécticos identificados, tendo em comum o seu esqueleto formado por unidades de ácido galacturônico (Figura 4): homogalacturonanas (HG), xilogalacturonanas (XG), ramnogalacturonanas do tipo I (RG-I) e ramnogalacturonanas do tipo II (RG-II) (WILLATS et al., 2006).

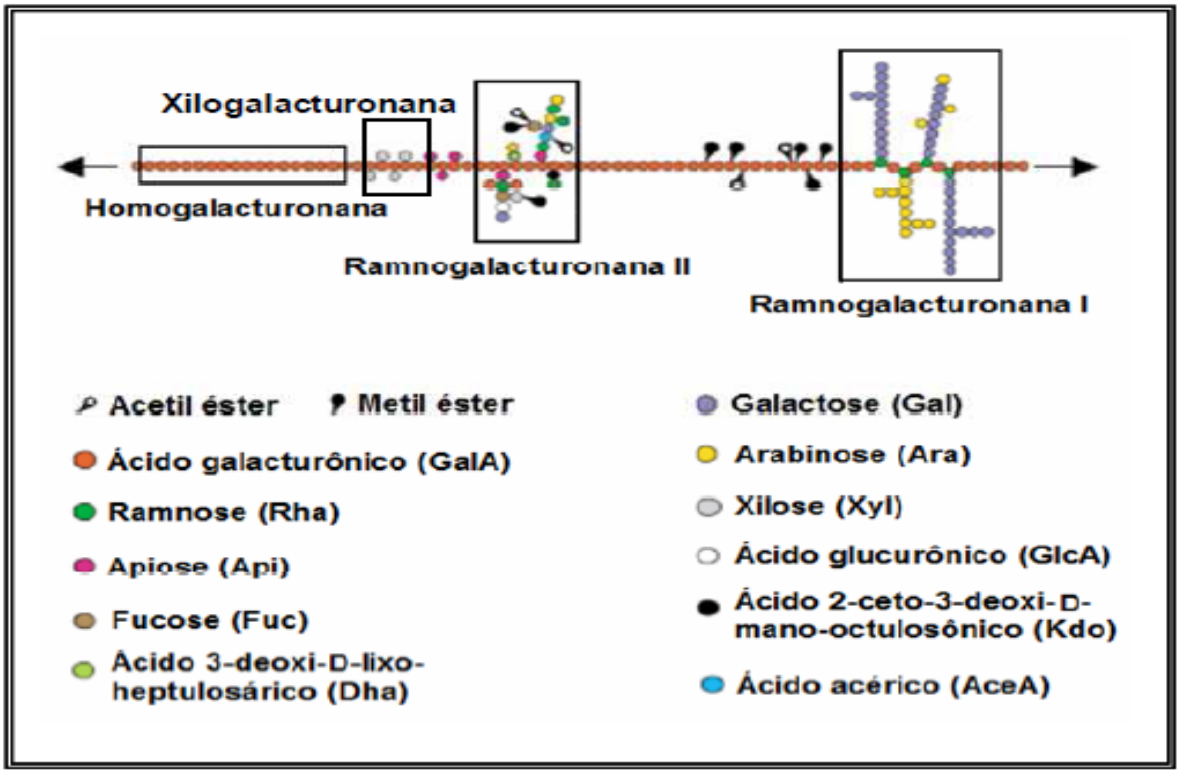


FIGURA 4. POLISSACARÍDEOS PÉCTICOS DA PAREDE CELULAR.
FONTE: adaptado de Vriesmann (2008).

As homogalacturonanas constituem a região lisa das pectinas, também conhecida como "*smooth region"* e são formadas por cadeias lineares de ácido α-D-galacturônico (1→4), que podem estar parcialmente metil-esterificados em C-6 e *O*-acetilados em C-2 e/ou C-3. Algumas unidades de ácido galacturônico podem aparecer substituídas em *O*-3 por unidades de xilose, formando as xilogalacturonanas (RIDLEY; O'NEILL; MOHNEN, 2001).

Ramnogalacturonanas são divididas em dois tipos: ramnogalacturonanas do tipo I (RG-I) e ramnogalacturonanas do tipo II (RG-II). As RG-I são formadas por uma cadeia principal de unidades alternantes de ácido α-D-galacturônico ligado (1→4), e α-L-ramnose ligada (1→2), à qual se ligam cadeias laterais de polissacarídeos neutros, tais como arabinanas, galactanas e arabinogalactanas (DE VRIES, 1988; SCHOLS; VORAGEN, 1996). Outros açúcares que podem estar ligados nas cadeias laterais são: D-xilose, D-glucose, D-manose, L-fucose e ácido D-glucurônico (VORAGEN et al., 1995).

As arabinogalactanas ocorrem em duas formas clássicas que estruturalmente são diferenciadas principalmente com relação ao tipo de ligação da cadeia principal. Arabinogalactanas do tipo I (AG-I) são constituídas por uma cadeia principal de β-D-galactopiranose (1→4)-ligada, substituída por unidades de arabinose e galactose piranosídica (STEPHEN, 1983). Entretanto, variações estruturais podem ocorrer, como verificado por Hinz et al. (2005), que identificaram a presença de elementos estruturais de β-D-galactopiranose (1→3)-ligada em AGI presentes na soja, laranja lima, batata e cebola, em diferentes proporções. As cadeias laterais presentes na AG-I são geralmente ligadas ao *O*-3 da cadeia principal de β-D-galactose e o conteúdo de arabinose que esta classe de arabinogalactanas pode apresentar é muito variável, podendo chegar a 50% (CARPITA; GIBEAUT, 1993). As unidades de arabinose nas ramificações da AG-I podem ser terminais ou ligadas em *O*-3, *O*-5 e *O*-3,5 (CIPRIANI et al., 2009a; DONG et al., 2007; HABIBI et al., 2004; IACOMINI et al., 2005; NERGARD et al., 2005) e mais raramente em *O*-2 e *O*-2,5 (CIPRIANI et al., 2009a; TISCHER; IACOMINI; GORIN, 2002).

Por outro lado, arabinogalactanas do tipo II (AG-II) são constituídas por uma cadeia principal formada por unidades de β-D-Gal*p* (1→3)-ligadas ou ainda β-D-Gal*p* (1→3) e (1→6)-ligadas (ASPINALL, 1969; FINCHER; STONE; CLARKE, 1983; LEIVAS; IACOMINI; CORDEIRO, 2016; MCNEIL et al., 1984; SIMAS-TOSIN et al., 2012; STEPHEN, 1983). As cadeias laterais podem estar inseridas nas posições *O*-3 e *O*-6 das unidades de β-D-Gal*p* que formam a cadeia principal (DONG; FANG, 2001; KANG et al., 2011; SAULNIER; BRILLOUET; JOSELEAU, 1988; WANG et al., 2003; WANG et al., 2005b). As ramificações incluem cadeias laterais constituídas por α-L-Ara*f* terminal, (1→3) e (1→5)-ligada. Além disso, ramificações podem conter unidades de α-D-Glc*p*A e 4-*O*-metil-α-D-Glc*p*A (ASPINALL, 1969;

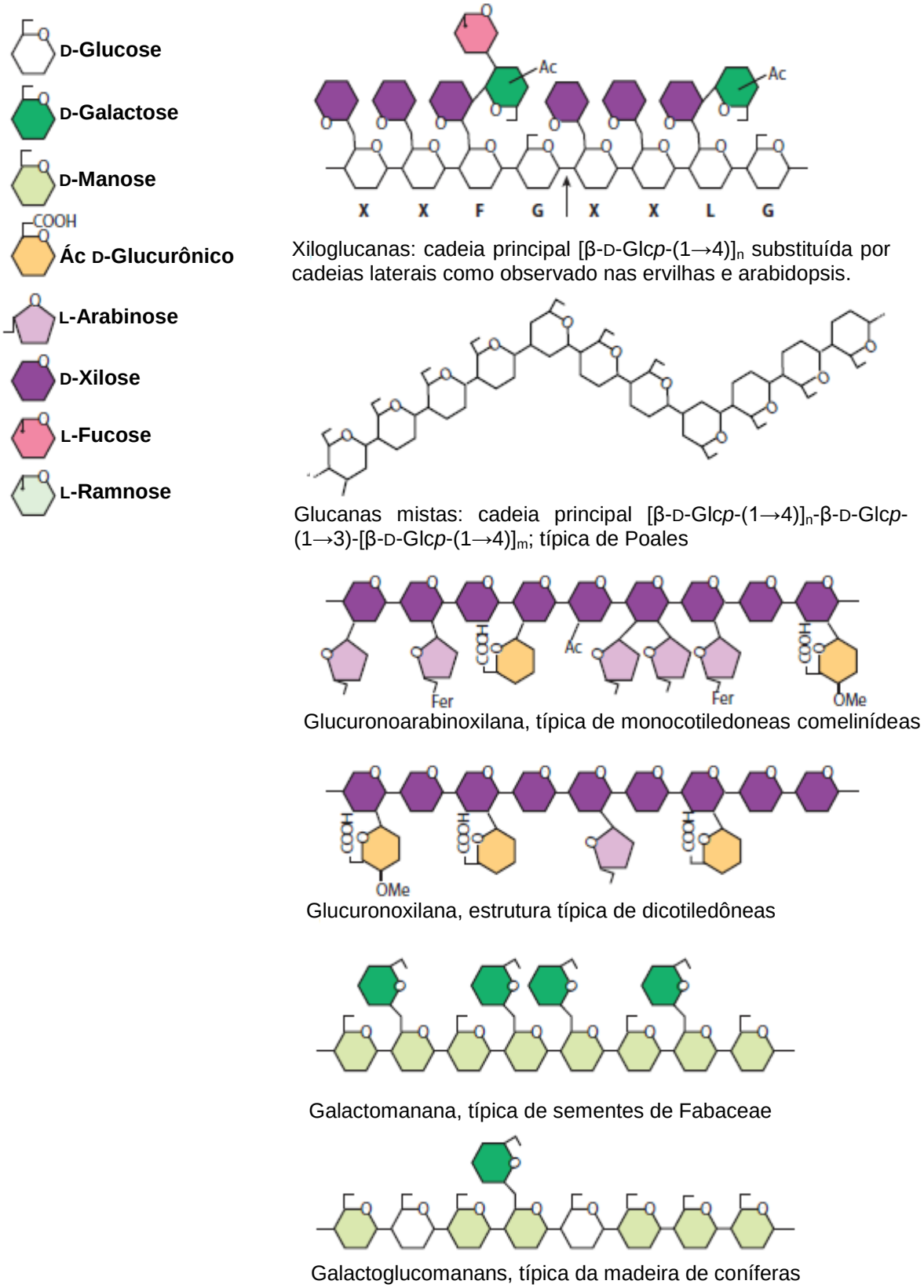
FINCHER et al., 1983; MCNEIL et al., 1984; STEPHEN, 1983; THUDE; CLASSEN, 2005).

Ramnogalacturonanas do tipo II são polissacarídeos pécticos bastante complexos, de baixo peso molecular, contendo na sua cadeia principal 7 a 10 unidades de α-D-Gal*p*A com ligação (1→4), substituídos em *O*-2 e/ou *O*-3 por cadeias laterais heteropoliméricas. Estas cadeias laterais das RG-II contêm cerca de 12 açúcares diferentes e 20 ligações distintas. Os açúcares mais comuns são L-Rha*p*, L-Ara*f*, D-Gal*p* e D-Glc*p*A. Outros açúcares incomuns como, por exemplo, apiose, ácido acérico e os açúcares metilados 2-*O*-metil-L-Fuc*p* e 2-*O*-metil-D-Xyl*p*, também podem ser encontrados nas RG-II. Estes polissacarídeos ocorrem em pequenas percentagens nas células do parênquima, mas têm uma função importante, especialmente como reguladores do crescimento e expansão da parede celular vegetal (VINCKEN et al., 2003).

2.3.2 Polissacarídeos Hemicelulósicos

A função principal das hemiceluloses em tecidos vegetais é unir microfibrilas de celulose, fortalecendo assim a parede celular (CARPITA; GIBEAUT, 1993; COSGROVE, 2005). Dessa forma, por estarem comumente associadas à celulose, hemiceluloses são normalmente extraídas da parede celular vegetal por meio de extrações alcalinas (ASPINALL, 1969; TIMELL, 1964).

O termo hemicelulose refere-se à um grupo heterogêneo de polissacarídeos e foi atribuído em uma época em que suas estruturas químicas ainda não eram bem compreendidas. A maioria dos autores atribui o termo como denominador comum de um grupo de polissacarídeos da parede celular caracterizado por não pertencer ao grupo das pectinas ou celulose e por possuir um esqueleto contendo glucose, manose ou xilose β-(1→4)- ligados, com C-1 e C-4 em posição equatorial (Figura 5) (SCHELLER; ULVSKOV, 2010; SPIRIDON; POPA, 2008).



Xiloglucanas: cadeia principal [β-D-Glc*p*-(1→4)]$_n$ substituída por cadeias laterais como observado nas ervilhas e arabidopsis.

Glucanas mistas: cadeia principal [β-D-Glc*p*-(1→4)]$_n$-β-D-Glc*p*-(1→3)-[β-D-Glc*p*-(1→4)]$_m$; típica de Poales

Glucuronoarabinoxilana, típica de monocotiledoneas comelinídeas

Glucuronoxilana, estrutura típica de dicotiledôneas

Galactomanana, típica de sementes de Fabaceae

Galactoglucomanans, típica da madeira de coníferas

FIGURA 5. POLISSACARÍDEOS HEMICELULÓSICOS DE PLANTAS E SUAS FONTES.

Abreviaturas: Fer – Ácido úlico;OMe – O-Metil; Ac – Acetil.

FONTE: adaptado de Scheller e Ulvskov (2010).

Estes polímeros podem estar ligados a uma variedade de cadeias laterais, assim como a grupamentos metil, acetil e ácido ferúlico (HARRIS; STONE, 2008; SCHELLER; ULVSKOV, 2010). As principais hemiceluloses encontradas em plantas incluem xiloglucanas, glucanas, (glucurono)arabinoxilanas, (glucurono)xilanas, galactomananas e galactoglucomanas (SCHELLER; ULVSKOV, 2010; SPIRIDON; POPA, 2008). Suas estruturas e fontes comuns estão representadas na Figura 5.

2.4 FRUTOS DE Arecaceae

Conforme descrito anteriormente no ítem 2.1, paredes celulares de diferentes tecidos de uma mesma planta podem apresentar uma mistura de polissacararídeos da parede celular primária e secundária. Neste contexto, células de frutos são conhecidas por conter majoritariamente polissacarídeos da parede celular primária (JOHN; DEY, 1986). Dessa forma, a avaliação da estrutura química de polissacarídeos de frutos da família Arecaceae pode ser usada como ferramenta para uma maior compreensão de relações filogenéticas que permeiam a composição da sua parede celular primária.

Países tropicais produzem uma grande quantidade de frutos pouco conhecidos, que são de potencial interesse para a indústria de alimentos. Frutos consumidos regionalmente tem ganhado popularidade devido aos sabores e cores diferenciados, e também ao valor nutricional e terapêutico atribuídos popularmente à alguns destes frutos. Enquanto as propriedades químicas e biológicas de frutos da Europa e América do Norte já estão bem descritos na literatura, os frutos nativos da América do Sul são menos estudados (CLERICI; CARVALHO-SILVA, 2011).

A Floresta Amazônica é considerada o maior reservatório natural da diversidade vegetal do planeta, sendo detentora de alta diversidade de espécies frutíferas ainda pouco estudadas (OLIVEIRA; AMARAL, 2004). Dentro dessa diversidade estão espécies da família Arecaceae, que além de contribuir com a produção de uma variedade de frutos comestíveis, podem produzir palmito (parte comestível do caule de algumas espécies), óleos, essências, ceras, bálsamos e

resina, além de possuir potencial paisagístico (BONDAR, 1964). Dentre os frutos da família Arecaceae encontrados na Amazônia destacam-se o buriti (*Mauritia flexuosa*), o tucumã (*Astrocaryum aculeatum*), o açaí (*Euterpe oleracea*) e a pupunha (*Bactris gasipaes*) que são objetos de estudo deste trabalho (Figura 6).

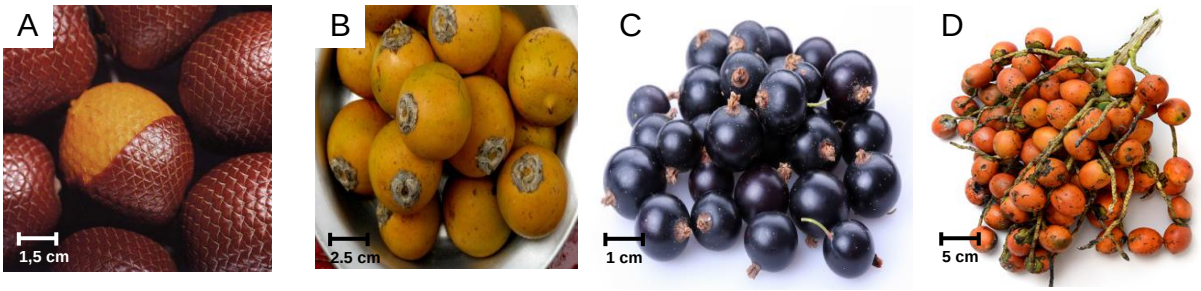


FIGURA 6. BURITI (A), TUCUMÃ (B), AÇAI (C), E PUPUNHA (D): FRUTOS DA FAMÍLIA ARECACEAE ENCONTRADOS NA AMAZÔNIA

FONTES - adaptado de:

A) https://ecodatainforma.wordpress.com/2012/04/03/abril-buriti/
B) http://www.pedromartinelli.com.br/blog/category/viagens/page/18/
C) http://www.portalviseu.com.br/
D) http://infograficos.estadao.com.br/paladar/cozinha-do-brasil/2013/ing-pupunha.html

Além do consumo regional, frutos do buriti e açaí estão entre as espécies nativas com maior potencial econômico na América Latina (BARRETO et al., 2012; TAVARES et al., 2003). Por outro lado, o tucumã e a pupunha, embora apresentem menor importância econômica que os frutos citados anteriormente, fazem parte da biodiversidade brasileira e são amplamente consumidos pela população local na região Amazônica e estão integrados aos seus hábitos alimentares (COSTA; VAN LEEUWEN; COSTA, 2005; MORA-URPÍ; WEBER; CLEMENT, 1997).

Com relação à composição química de macronutrientes, buriti, tucumã, açaí e pupunha são ricos em carboidratos e lipídeos conforme demonstrado na Tabela 1.

Tabela 1- Composição de macronutrientes* presentes no buriti, tucumã, açaí e pupunha

	Carboidratos (%)	Proteínas (%)	Lipídeos (%)
Buriti	43,0	3,8	53,2
Tucumã	5,8	4,6	89,6
Açai	87,6	5,5	6,90
Pupunha	26,8	4,0	69,2

*Valores médios, baseados no peso seco dos frutos.

FONTE: Aguiar et al. (1980).

Entretanto, a descrição a cerca da estrutura destes carboidratos, em especial da porção de fibras dietéticas, e suas interações com a microbiota intestinal humana são pouco conhecidas.

2.5 POLISSACARÍDEOS COMO FIBRAS DIETÉTICAS E SEU POTENCIAL FUNCIONAL FISIOLÓGICO

Fibras ditéticas são polímeros de carboidratos com três ou mais unidades monoméricas, e a lignina (um polímero de fenilpropano), resistentes à digestão por enzimas produzidas pelo homem no trato gastrointestinal superior, chegando ao colon em um estado químico inalterado, onde podem ser fermentados pela microbiota colônica. Dessa forma, pectinas e hemiceluloses enquadram-se na categoria de fibras dietéticas (JONES, 2014; BERNAUD; RODRIGUES, 2013).

Biologicamente, fibras dietéticas desempenham diversas funções. Por este motivo, um consumo diário adequado de fibras está entre 30-38 g para homens e 21-25 g para mulheres adultas variando conforme a faixa etária (TRUMBO et al., 2002). Entre os benefícios associados ao alto consumo de fibras estão a redução do risco de doenças como o câncer colorretal, doença coronariana, aumento da saciedade, controle dos níveis plasmáticos pós-prandial de glucose, insulina, lipídios

e colesterol, regulação do trânsito intestinal, prevenção de patologias associadas à disbiose intestinal, entre outros (KACZMARCZYK; MILLER; FREUND, 2012).

Em geral, fibras dietéticas podem ser divididas em dois grupos de acordo com sua capacidade de solubilização: fibras dietéticas solúveis e insolúveis. Fisiologicamente, fibras insolúveis tem conhecido efeito laxativo, aumentanto o volume fecal e diluindo metabólitos tóxicos no intestino enquanto fibras solúveis são conhecidas por retardar o trânsito gastrointestinal/absorção de nutrientes e sofrer fermentação pela microbiota intestinal no colon gerando benefícios para o hospedeiro (PERIAGO; ROS; LÓPEZ, 1993). Entretanto, diversos estudos têm indicado que mesmo as fibras insolúveis são passíveis de fermentação pela microbiota intestinal humana em maior ou menor grau (HAMAKER; TUNCIL, 2014).

2.5.1 A microbiota intestinal e metabólitos produzidos durante fermentação bacteriana

O trato gastrointestinal humano contém aproximadamente 10^{14} microorganismos, majoritariamente bactérias, que pertencem a cerca de 1.000 espécies distintas (EGERT et al., 2006). A maior parte dessas bactérias é encontrada no colon, totalizando cerca de 1-2 kg de peso. Quatro filos bacterianos principais compõe a microbiota colônica: Firmicutes, Bacteroidetes, Actinobacterias e Proteobacterias. A inclusão do gênero *Clostridium* no filo dos Firmicutes, faz deste o filo mais abundante de bactérias intestinais, constituindo 46-58% da microbiota intestinal total. Outros grandes grupos de bactérias como *Bacteroides* e *Prevotella* (gêneros pertentencentes ao filo dos Bacteroidetes), perfazem 10-30% do total de bactérias, *Bifidobacterias* (gênero pertentencente ao filo das Actinobacterias) 4.4–4.8%*, Enterobacterias* (gênero pertentencente ao filo das Proteobactérias) 0.1–0.2% e *Lactobacillus* e *Enterococcus (*gêneros pertentencentes ao filo dos Firmicutes) ambos entre 0.1–1.8% (PAYNE et al., 2012). De maneira geral, a microbiota intestinal está envolvida em diversas funções fisiológicas, como na modulação da biodisponibilidade de nutrientes, produção de vitaminas (principalmente K e

complexo B), inibição da colonização por patógenos, remoção de compostos nocivos ao corpo humano e degradação de compostos não digeríveis como fibras dietéticas (HOOPER; MIDTVEDT; GORDON, 2002; SAAD, 2006).

Os microorganismos presentes no intestino são capazes de hidrolisar os polissacarídeos não digeríveis por enzimas produzidas pelo homem (por exemplo amido resistente e a fibra dietética solúvel e insolúvel) e proteínas ou mucinas do hospedeiro (proteínas de elevado peso molecular glicosiladas). A fermentação de polissacarídeos resulta na produção de ácidos graxos de cadeia curta (AGCC), lactato, formato, etanol e misturas de gases como CH_4, CO_2 e H_2 (KHANNA; PARRETT; EDWARDS, 2006). O metabolismo de material protéico produz ácidos graxos de cadeia ramificada (AGCR), amônia, aminas, mercaptanos e H_2S, bem como alguns compostos indólicos e fenólicos tóxicos. A produção de AGCC resultante da fermentação microbiana no intestino satisfaz cerca de 10% do total dos requerimentos energéticos diários do homem. Além disso, AGCC aumentam a solubilidade e biodisponibilidade de minerais como cálcio, fósforo e magnésio (STEVENS; HUME, 1998). Acetato, propionato e butirato são os principais AGCC produzidos pelas bactérias intestinais, típicamente em proporção de ~3:1:1, e cada um deles desempenha funções únicas relacionadas à saúde do hospedeiro (CUMMINGS, 1981; TOPPING; CLIFTON, 2001). O acetato é o AGCC que atinge maior concentração plasmática e é metabolizado nos músculos, rins, coração e cérebro onde pode ser usado como fonte energética. Também auxilia no controle de inflamação, invasão por patógenos, e estudos *in vitro* demonstram que juntamente com lactato, está envolvido na proliferação celular (FUKUDA et al., 2011; MASLOWSKI et al., 2009; MATSUKI et al., 2013). O butirato é a fonte energética preferida por colonócitos, tendo papel importante na manutenção da integridade da barreira intestinal. Atua promovendo diferenciação celular, na supressão da inflamação do colon, e é capaz de inibir o ciclo celular e provocar apoptose em células cancerígenas colônicas (CANANI et al., 2011). O propionato é metabolizado no fígado onde possivelmente é utilizado como substrato gluconeogênico e apresenta capacidade de inibir a produção de colesterol endógeno (GIBSON, 1999). Mais recentemente, foi demonstrado que o propionato está relacionado a mecanismos que podem influenciar a saciedade, prevenir obesidade induzida por dieta e melhorar a sensibilidade à insulina (CHAMBERS et al., 2014; NØHR et al.,

2013). Dessa forma, AGCC estão relacionados à prevenção de diversas patologias como alergias, doenças inflamatórias intestinais, câncer e diabetes, sendo cada vez mais elucidada a sua relação também com doenças cardiovasculares e dislipidemias (BESTEN et al., 2013).

Por outro lado, derivados de fermentação protéica como AGCR, amônia, indois e fenóis recebem atenção devido ao seu caráter tóxico. A amônia induz a uma aceleração na renovação celular e geralmente é mais tóxica para células saudáveis do que transformadas, o que pode selecionar linhagens cancerígenas. O crescimento bacteriano estímulado pela fermentação de carboidratos no intestino por sua vez, reduz a concentração de amônia no lúmen, devido ao maior requerimento bacteriano por nitrogênio. Compostos fenólicos e indólicos são resultantes da deaminação de aminoácidos aromáticos e são normalmente detoxificados por conjugação com glucuronideos ou sulfatos no fígado e excretados na urina. Quando não detoxificados, indois e fenois atuam como pró-carcinogênicos (CUMMINGS; MACFARLANE, 1991).

2.5.2 Modulação da microbiota intestinal por fibras dietéticas – prebióticos

O termo "prébiótico" foi definido pela primeira vez como "um ingrediente alimentar não digerível que afeta beneficamente o hospedeiro ao estimular seletivamente o crescimento e/ou atividade de um ou um número limitado de bactérias no colon" (GLENN; ROBERFROID, 1995). Alterações na população bacteriana intestinal e consequente produção de metabólitos podem ser moduladas por fatores dietéticos, em especial fibras dietéticas (BESTEN et al., 2013). Entretanto, pouco é conhecido a respeito de como fibras dietéticas prebióticas podem alterar a atividade e composição da microbiota intestinal de maneira preditiva (HAMAKER; TUNCIL, 2014).

Para tanto, a compreensão das preferências bacterianas por cada tipo de estrutura química se faz necessária. Isso porque nem todas as bactérias possuem as mesmas habilidades para digerir diferentes fibras dietéticas. Cada bactéria possui

“clusters” genéticos que codificam para a produção de enzimas específicas que degradam ligações entre monossacarídeos característicos de um carboidrato (*Carbohydrate Active Enzymes* - “*CaZymes*”, em inglês). Dessa forma, dependendo da estrutura química das fibras dietéticas presentes no intestino, um determinado grupo de bactérias equipado com enzimas equivalentes ao polímero presente serão favorecidas em detrimento daquelas que não possuem tal maquinaria (HAMAKER; TUNCIL, 2014).

Em geral, mesmo polissacarídeos com estruturas muito simples com apenas um tipo de monossacarídeo e um ou dois tipos de ligação entre açúcares, como é o caso das frutanas (essencialmente frutose com ligações β-(1→2)), podem favorecer grupos específicos de bactérias (HERNOT et al., 2009; LESMES et al., 2008). Devido à baixa complexidade destes polímeros, diversas bactérias intestinais possuem enzimas necessárias à sua fermentação. Entretanto, no ambiente competitivo do colon intestinal, bactérias desenvolveram preferências específicas relacionadas, por exemplo, a estrutura tridimensional do amido e ao tamanho das frutanas. Dessa forma, bactérias distintas apresentam competitividade e especificidade mesmo para estas fibras de estrutura relativamente simples (HAMAKER; TUNCIL, 2014).

Por outro lado, carboidratos mais complexos como pectinas (rhamnogalacturonanas, arabinogalactanas, etc.) e hemiceluloses (xiloglucanas, arabinoxilanas, galactomananas, etc.), podem conter inúmeros monossacarídeos distintos e vários tipos de ligações. Adicionalmente, cada um desses polímeros pode apresentar diversas variações dependendo da sua fonte, grau de solubilidade, arranjo com outros polímeros, estrutura tridimensional e estrutura química fina. Conforme discutido anteriormente no ítem 2.3, o tamanho do polissacarídeo, a presença de grupamentos metil e acetil e distribuição e tamanho de cadeias laterais, variam dentro de uma mesma classe de moléculas (IZYDORCZYK; BILIADERIS, 1995; MOHNEN, 2008; SAULNIER et al., 2007; SCHELLER; ULVSKOV, 2010; THAKUR et al., 1997). Dessa forma, características de cada tipo de estrutura química permitem que uma determinada espécie bacteriana tenha vantagem competitiva na fermentação da mesma (HAMAKER; TUNCIL, 2014).

Isto pode explicar resultados conflitantes dos efeitos de uma mesma classe de fibras sob a microbiota intestinal. Por exemplo, em um estudo de três semanas foi observado um aumento na abundância de *Bacteroides* spp. no colon de ratos alimentados com dieta contendo 6,5% de pectinas cítricas (DONGOWSKI; LORENZ; PROLL, 2002), enquanto que um estudo de 4 semanas com ratos que recebram 7% de pectinas da maçã, apresentaram aumento de gêneros de *Anaeroplasma*, *Anaerostipes* e *Roseburia* e redução no número de *Alistipes* e *Bacteroides* spp (LICHT et al., 2010). Isso porque diferenças na fonte e métodos de extração destas pectinas resultam em diferenças na sua estrutura química (como o tamanho, teor de metilesterificação e presença de cadeias laterais) que influenciam a subsequente utilização de polímeros pela microbiota.

Dentre os carboidratos mais estudados com efeitos prebióticos estão os carboidratos solúveis e de estruturas mais simples como frutooligossacarídeos e a inulina extraída de plantas (KAJIWARA; GANDHI; USTUNOL, 2002). Algumas frutas como kiwi, maçã e *Mangifera pajang*, já foram estudadas com relação à sua atividade prebiótica (AL-SHERAJI et al., 2012; BLATCHFORD et al., 2015; MEHRLÄNDER et al., 2002). Entretanto, relações entre a estrutura química fina dos seus polissacarídeos e/ou outros compostos e seu perfil fermentativo pela microbiota intestinal humana não são bem compreendidos.

O grupo de química de carboidratos da Universidade Federal do Paraná há 51 anos investiga a estrutura química de polissacarídeos de diversas fontes e, mais recentemente, tem buscado relacionar componentes estruturais de carboidratos com suas atividades biológicas. Entretanto, investigações a respeito da ação de polissacarídeos sob a microbiota intestinal humana não havia sido investigada até o momento em nosso grupo de pesquisa. Além disso, o conhecimento das estruturas químicas de polissacarídeos do buriti, tucumã, açaí e pupunha e avaliação de seu potencial prebiótico agregam valor comercial e nutricional a estes frutos brasileiros.

3. OBJETIVOS

3.1 OBJETIVO GERAL

Caracterizar estruturalmente polissacarídeos presentes nos frutos do buriti (*Mauritia flexuosa*), açaí (*Euterpe oleracea*), tucumã (*Astrocaryum aculeatum*) e pupunha (*Bactris gasipaes*) e avaliar seus perfis de fermentação pela microbiota intestinal humana.

3.2 OBJETIVOS ESPECÍFICOS

Para atingir o objetivo geral acima descrito, os seguintes objetivos específicos foram delineados:

- Extrair polissacarídeos da polpa do buriti, tucumã, açaí e pupunha;
- Purificar e caracterizar estruturalmente os polissacarídeos obtidos;
- Com base nas estruturas químicas dos polissacarídeos encontrados, selecionar e avaliar o perfil de fermentação *in vitro* pela microbiota intestinal humana dos polissacarídeos de diferentes estruturas químicas.

ARTIGO I

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Arabinan-rich pectic polysaccharides from buriti (*Mauritia Flexuosa*): an Amazonian edible palm fruit

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ABSTRACT

Primary cell wall polysaccharides from aqueous extract of buriti fruit pulp (*Mauritia flexuosa*, an exotic tropical palm) were isolated and characterized. After freeze–thaw and α-amylase treatments, extracted polysaccharides were purified by sequential ultrafiltration through membranes. Two homogeneous fractions were obtained, SBW-100R and SBW-30R (M_w of 126 kDa and 20 kDa, respectively). Monosaccharide composition, methylation and ^{13}C-NMR analysis showed that fraction SBW-100R contained a (1→5)-linked arabinan, branched at *O*-3 and *O*-2 positions, linked to a type I rhamnogalacturonan. Low amounts of these polymers were also present in fraction SBW-30R according to ^{13}C-NMR analysis and monosaccharide composition. However, a high methyl esterified homogalacturonan (HG) was present in higher proportions. These results reinforce previous findings present in literature data which indicate that pectic polysaccharides are found in high amounts in primary cell walls of palms, which are commelinid monocotyledons.

Keywords: *Mauritia Flexuosa*, buriti fruits, pectic polysaccharides, arabinan, type I rhamnogalacturonan, homogalacturonan.

1. Introduction

The Amazonian region is considered the biggest natural reservoir of vegetal diversity in the planet and houses a large variety of fruit crops, some of which have potentially promising health and nutritional properties (Oliveira & Amaral, 2004). Buriti (*Mauritia flexuosa*), belonging to the Arecaceae family, is a palm widely distributed in the Amazon Rain Forest of Brazil (Delgado, Couturier & Mejia, 2007). The tree has been used for centuries by the Native population, and has a high ecological, cultural and economic value (Manhães, 2007; Tavares et al., 2003). The fruits are largely consumed by the local population and are considered one of the best sources of pro-vitamin A found in the Brazilian biodiversity (Ministério da Saúde, 2002). Regarding to its macronutrients, the pulp *in natura* of buriti fruits is mainly composed by carbohydrates and lipids (25.53% and 18.16%, respectively) (Carneiro & Carneiro, 2011).

Carbohydrates and glycosylated compounds play an important role in determining specific characteristics of the fruit such as color, texture and flavor, and, moreover, can have different biological applications according to their structures (Kumar, Sinha, Makkar, de Boeck, & Becker, 2012; Ladaniya, 2008).

Few studies investigated cell wall polysaccharides from palm fruits. Palms belong to the Arecaceae family, which is a monocotyledon of the commelinid group. Most research on the primary cell walls of the commelinid group has focused on the walls of the Poaceae (grasses and cereals) and indicate that the most abundant non-cellulosic polysaccharides are galactoarabinoxylans rather than pectic polysaccharides with variable proportions of (1→3),(1→4)-β-D-glucans (Harris, 2005; Harris, Kelderman, Kendon & Mckenzie, 1997). However, in contrast to the walls of the Poales, the walls of the Arecaceae, which is a basal family in the commelinid group, seems to have a similar composition to the walls of dicotyledons and non-commelinid monocotyledons, with large proportions of pectic polysaccharides and smaller amounts of xyloglucans (Carnachan & Harris, 2000; Harris, 2005).

It has recently been shown the presence of linear polysaccharides in the pulp of buriti, namely a (1→3),(1→4)-α-D-glucan (1:4), a (1→5)-linked α-L-arabinan and a (1→4)-linked β-D-xylan (Cordeiro, de Almeida & Iacomini, 2015). However, the presence of water extractable pectic polysaccharides in buriti has not been investigated so far.

Once the polysaccharide's physicochemical properties play a critical role in governing its physiological effects, information about polysaccharides composition and organization is fundamental to understand many nutritional and technological aspects of fruits (Ladaniya, 2008). Moreover, structural characterization of polysaccharides present in buriti aqueous extract may add more insights into the taxonomic division of Arecaceae family and its relation to primary cell wall composition.

2. Materials and methods

2.1 Plant material

Ripe fruits of buriti (*M. flexuosa*) were purchased at local market in the Cruzeiro do Sul city, State of Acre, Brazil.

2.2 Extraction and purification of polysaccharides

The cold-water soluble fraction from buriti, SBW-A, was obtained as previously described by Cordeiro et al. (2015). Fraction SBW-A was later purified by sequential ultrafiltration through membranes with cut-offs of 300 kDa, 100 kDa and 30 kDa (Ultracel, Millipore). All the extraction and purification procedures are summarized in Figure 1.

The yields were expressed as % based on the weight of dried buriti pulp that was submitted to extraction (55 g).

2.3 Sugar composition

Neutral monosaccharide components of the polysaccharides and their ratio were determined by hydrolysis with 2 M TFA for 8 h at 100°C, followed by conversion to alditol acetates by successive $NaBH_4$ reduction and acetylation with Ac_2O-pyridine (1:1, v/v, 1ml) at room temperature for 14h, and the resulting alditol acetates extracted with $CHCl_3$. These were analyzed by GC–MS using a Varian Saturn 2000R model 3800 gas chromatograph linked to a Varian Ion-Trap 2000R mass spectrometer, with He as carrier gas. A capillary column (30 m x 0.25 mm i.d.) of DB-225, hold at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 220 ºC and hold at this constant temperature for 19.75 min was used for the quantitative analysis.

Uronic acid contents were determined spectrofotometrically using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

2.4 Methylation analysis of polysaccharides

The polysaccharides from SBW-100R were *O*-methylated according to the method of (CIUCANU; KEREK, 1984), using powdered NaOH in DMSO-MeI. The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 12 h) and neutralization with $BaCO_3$. The material was then submitted to reduction and acetylation as described above for sugar composition, except that the reduction was performed using $NaBD_4$. The products (partially *O*-methylated alditol acetates) were examined by capillary GC-MS. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50

ºC during injection for 1 min and then programmed at 40 ºC/min to 210 ºC and held at this temperature for 31 min, was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (SASSAKI; IACOMINI; GORIN, 2005b).

2.5 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C {^{1}H} and DEPT-135 NMR spectra were acquired at 50°C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The samples were acquired in D_2O with chemical shifts expressed as δ PPM, using the resonances of CH_3 groups of acetone (δ 30.2) as internal reference.

The degree of methyl esterification (DE) was determined by ^{1}H NMR spectroscopy according Grasdalen, Bakoy & Larsen (1988). Briefly, the fraction was deuterium-exchanged three times by freeze-drying with D_2O solutions, finally dissolved in D_2O, transferred into 5-mm NMR tube. The ^{1}H NMR spectra were acquired at 70°C, with 256 scans, on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{1}H at 400.13 MHz. Chemical shifts were expressed as δ PPM, using the resonances of HDO at δ 4.22 as internal reference. All pulse programs were supplied by Bruker.

2.6 Determination of homogeneity and molecular weight of polysaccharides

The homogeneity and molecular weight (M_w) of water soluble polysaccharides were determined by high performance size exclusion chromatography (HPSEC), using a Waters 2410 differential refractometer and a Pharmacia LKB Uvicord VW 2251 ultraviolet detector at 280 nm (UV) as detection equipments. Four columns were used in series, with exclusion sizes of 7 x 10^6 Da (Ultrahydrogel 2000, Waters),

4 x 10^5 Da (Ultrahydrogel 500, Waters), 8 x 10^4 Da (Ultrahydrogel 250, Waters) and 5 x 10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. The samples, previously filtered through a membrane (0.22 µm, Millipore), were injected at a concentration of 1 mg/ml, at room temperature. The specific refractive index increment (*dn/dc*) was determined and the results was processed with software ASTRA provided by the manufacturer (Wyatt Technologies).

3. Results and discussion

The dried pulp powder (55 g) of buriti fruits was defatted and the polysaccharides (fraction BW, 12% yield) extracted with water (at 100ºC) as previously described by Cordeiro et al. (2015). Fraction BW was submitted to freeze-thawing and α-amylase treatments, giving rise to fraction SBW-A, in 4.6% yield. This was mainly composed by arabinose and uronic acid (Table 1), indicating the presence of pectic polysaccharides (Cordeiro et al., 2015).

When analyzed by HPSEC, SBW-A demonstrated a heterogeneous profile (Fig. 2A), and thus was further fractionated by sequential ultrafiltration through membranes with cut-offs of 300 kDa, 100 kDa and 30 kDa (Fig. 1). According to HPSEC, fractions retained in 100 kDa and 30 kDa (SBW-100R and SBW-30R, respectively) contained homogeneous polysaccharides (Fig. 2 B), with average molar mass (M_w) of 126 kDa (*dn*/*dc* = 0.194) and 20 kDa (*dn*/*dc* = 0.213), respectively

Fraction SBW-100R was also mainly composed by arabinose and uronic acids, together with small amounts of galactose and rhamnose (Table 1), indicating the presence of an arabinan-rich pectic polysaccharide.

In order to achieve the linkages present in this arabinan-rich pectic polysaccharide, fraction SBW-100R was carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972) and submitted to methylation analysis (Table 2). The main observed derivatives were those of arabinose (55.9%), which was in

agreement with monosaccharide analysis. It could be seen the presence of high amounts of 2,3-Me_2-Ara-ol acetate, indicating the presence of (1→5)-linked Ara*f* units. The presence of (1→4)-linked Ara*p* units is ruled out by NMR results showed below, which demonstrated the presence of low-field chemical shifts of anomeric carbons of Ara*f* units). The presence of 3-Me- and 2-Me-Ara-ol acetates indicated that this arabinan was branched at *O*-2 and *O*-3, respectively. The derivative 2,5-Me_2-Ara-ol acetate indicated (1→3)-linked Ara*f* units, probably present as side chains. Terminal arabinopyranose was also observed in small amounts (2.6%). This arabinan is probably attached in the *O*-4 position of a type I rhamnogalacturonan (RG-I) as the derivatives of 2,4-di-*O*- and 4-*O*-linked rhamnopyranose were also found in methylation analysis (Table 2). The proportion of the derivatives 2,4-di-*O*- and 4-*O*-linked rhamnopyranose indicated that about 45% of rhamnose units were substituted by branching chains. The (1→4)-linked Gal*p*A which is interspersed with the rhamnopyranose units in type I rhamnogalacturonan, was also present as could be seen by the derivative 2,3,6-Me_3-Gal-ol-acetate. The (1→4)-linked Gal*p*A could also be branched at *O*-2 and *O*-3 in low amounts, due to the presence of the derivatives 3,6-Me_2-Gal-ol-acetate and 2,6-Me_2-Gal-ol-acetate, respectively. Moreover, other galactose derivatives were found in methylation analysis in minor proportions (Table 2), such as 2,3,4-Me_3-Gal-ol-acetate, 2,3-Me_2-Gal-ol-acetate and 2,4-Me_2-Gal-ol-acetate, indicating the presence of 6-*O*-, 4,6-di-*O*- and 3,6-di-*O*-substituted Gal*p* units, probably arising from a galactan.

The NMR analysis (^{13}C and HSQC) of SBW-100R is in accordance with composition and methylation analysis, being dominated bythe Araf signals (Fig. 3A and Fig. 4A). The low-field chemical shifts of anomeric carbons at δ 107.5/5.08, 107.1/5.17 and 106.4/5.24 confirmed that L-Ara adopted mainly α configuration and the furanose form. The^{13}C DEPT NMR spectrum showed a downfield shifted sig-nal at δ 66.2, corresponding to substituted C-5 from α-L-Ara*f* units,while the inverted signals at δ 62.7 and 61.2 can be assigned to unsubstituted C-5 (Cordeiro et al., 2012; Dourado, Cardoso, Silva, Gama & Coimbra, 2006; Navarro, Cerezo & Stortz, 2002). According to literature, the signal at δ 103.6/4.46 corresponded to C-1/H1 of β-D-Gal*p* units (Wagner & Jordan, 1988; Thude & Classen, 2005). The ^{13}C DEPT NMR spectrum showed a downfield shifted C-6 signal at δ 69.4 (Fig. 3A) and confirmed the presence of (1→6)-linked Gal*p* units. This signal coupled with its

hydrogen at δ 4.02 (Fig. 4A). The signals of C-1 from the rhamnogalacturonan core appeared at δ 99.5 and 97.9 from β-D-Gal*p*A and α-L-Rha*p* units, respectively, and the presence of C-6 of Rha*p* units at δ 16.7.

Thus, the above results suggested that fraction SBW-100R contains a branched arabinan which is linked to a type I rhamnogalacturonan.

Regarding fraction SBW-30R, it also contained arabinose and uronic acids as main monosaccharides, but in contrast to what was found in fraction SBW-100R, the content of uronic acid was greater than arabinose (Table 1). Indeed, the ^{13}C-NMR spectrum of SBW-30R showed low intensity anomeric signals of Ara*f* units at δ 107.6, 107.0 and 106.5. The C-6 signal of α-L-Rha*p* units from the rhamnogalacturonan could also be seen at δ 16.8. However, intense signals at δ 100.1 and 99.3, corresponding to anomeric carbons of esterified and unesterified units of α-D-Gal*p*A, respectively, are present in this fraction (Westereng, Michaelsen, Samuelsen & Knutsen, 2008). The C-6 signal from esterified carbonyl carbons could be seen at δ 170.6. Signals of acetyl carbons and methyl carbons of esterified carbonyls in Gal*p*A units appeared at δ 20.4 and 52.8, respectively. The remaining carbons of the α-D-Gal*p*A ring were seen at δ 78.7 (*O*-substituted C-4), δ 70.5 (C-5), δ 68.2 (C-3) and δ 68.0 (C-2). These assignments are in agreement with published literature data (Cantu-Jungles et al., 2014; Li, Cui, Nie & Xie, 2014). These spectral data indicated the presence of a pectin complex in fraction SBW-30R, probably a methyl esterified homogalacturonan (HG) together with an arabinan linked to a type I rhamnogalacturonan.

Due to the presence of methyl esterified α-D-Gal*p*A units in SBW-30R, the degree of methyl esterification (DE) was determined by ^{1}H NMR spectroscopy. A value of 75% was found, characterizing this polymer as high-methoxyl (HM) pectin.

In order to estimate the molar proportion of homogalacturonan relative to the type I rhamnogalacturonan copolymers chains in both fractions (SBW-100R and SBW-30R) the ratio HG/RG-I(%) was roughly estimated using the following relation, from a previously published work (Koffi, Yapo & Besson, 2013):

$$HG/RG\text{-}I(\%) = 100 \times \frac{[GalA(\%) - Rha(\%)]}{[2Rha(\%)+Ara(\%)+Gal(\%)]}$$

The HG to RG-I (%) ratio in fraction SBW-30R was 83% while in fraction SBW-100R was only 22%, indicating a larger homogalacturonan portion in fraction SBW30R, which is in agreement with the results demonstrated above.

The ratio of (Ara+Gal) to Rha is employed to estimate the relative importance of the neutral side chains to the rhamnogalacturonan backbone. The lower this ratio, the shorter the side chains attached to 4-*O*-Rha in RG-I backbone (Renard & Ginies, 2009; Yu et al., 2010). The calculated ratios for fractions SBW-100R and SBW-30R were 9.7 and 4.4, respectively, demonstrating that rhamnogalacturonan in fraction SBW-100R had higher amounts of neutral side chains than fraction SBW-30R.

Thus, as seen from monosaccharide composition and ^{13}C-NMR spectroscopy, SBW-100R and SBW-30R differ mainly in their molecular mass and the content of homogalacturonan/rhamnogalacturonan present in the pectic domain. Moreover, the RG-I present in these fractions had different proportions of neutral sugar side chains, especially arabinans.

There are few reports about polysaccharides that compose the primary cell walls of palms and this is the first report in the literature on structural elucidation of pectins from palm fruits. For buriti fruits, Cordeiro et al. (2015) have already reported the presence of linear polymers, a (1→3),(1→4)-α-D-glucan (1:4), a (1→5)-linked α-L-arabinan and a (1→4)-linked β-D-xylan. The presence of a branched α-glucan was reported for fruits of the palm *Orbignya phalerata* (Silva & Parente, 2001), while the heteropolysaccharides galactoglucomannan and galactomannoglucan were reported for fruits of *Acrocomia aculeata* (Silva, Silva & Parente, 2009), of *Arecastrum romanzoffianum* (Silva & Parente, 2010a) and of *Syagrus oleracea* (Silva & Parente, 2010b).

The present study also confirmed the results previously obtained from the composition of primary cell walls from the palms *Phoenix canariensis* and *Rhopalostylis sapida* (Carnachan & Harris, 2000), where pectic polysaccharides with the arabinan moiety were found. Moreover, this study contributes to the hypothesis

that members of the family Arecaceae possess high amounts of pectic polysaccharides like the members of non-commelinid and dicotyledon families. It also reinforces the evolutionary theory in the taxa of Monocotyledons (Carnachan & Harris, 2000), where the composition of palms primary cell walls may represent a transition from the composition of cell walls of non-commelinid families to commelinid families less derived than the Poales.

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Table 1- Monosaccharide composition of fractions obtained from the aqueous extract of buriti fruits (*M. flexuosa*).

Fractions	Monosaccharide composition (mol %)						UA[a] (%w/w)
	Rha	Ara	Xyl	Man	Gal	Glc	
SBW-A[b]	5.2	72	9	2.8	8	3	25
SBW-100R	6.9	60.3	-	-	6.9	-	25.9
SBW-30R	8.4	30.8	-	-	6.9	-	53.9

[a] Uronic acids, determined using the m-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

[b] From Cordeiro et al. (2015).

Table 2 - Linkage types based on analysis of partially *O*-methylalditol acetates obtained from methylated fraction SBW-100R from the pulp of buriti (*M. flexuosa*).

Partially *O*-methylalditol acetate	SBW-100R mol % [b]	SBW-100R %	Linkage type[c]
Arabinose			
2,3,5-Me_3-Ara[a]	12.5	22.4	Ara*f*-(1→
2,3,4-Me_3-Ara	2.6	4.7	Ara*p*-(1→
2,5-Me_2-Ara	5.0	8.9	→3)-Ara*f*-(1→
2,3-Me_2-Ara	19.5	34.9	→5)-Ara*f*-(1→
2-Me-Ara	9.8	17.5	→3,5)-Ara*f*-(1→
3-Me-Ara	6.5	11.6	→2,5)-Ara*f*-(1→
	55.9	100.0	
Galactose			
2,3,4,6-Me_4-Gal	13.4	39.6	Gal*p*-(1→
2,3,6-Me_3-Gal	9.3	27.5	→4)-Gal*p*-(1→
2,3,4-Me_3-Gal	4.8	14.2	→6)-Gal*p*-(1→
2,6-Me_2-Gal	2.4	7.1	→3,4)-Gal*p*-(1→
3,6-Me_2-Gal	0.7	2.1	→2,4)-Gal*p*-(1→
2,3-Me_2-Gal	0.8	2.4	→4,6)-Gal*p*-(1→
2,4-Me_2-Gal	2.4	7.1	→3,6)-Gal*p*-(1→
	33.8	100.0	
Rhamnose			
3,4-Me_2-Rha	5.6	54.4	→2)-Rha*p*-(1→
3-Me-Rha	4.7	45.6	→2,4)-Rha*p*-(1→
	10.3	100.0	

[a] 2,3,5-Me3-Ara = 2,3,5-tri-O-Methylarabinitolacetate, etc. b Quantified according to their effective carbon response (Sweet, Shapiro & Albersheim, 1975). Samples were carboxy-reduced by the carbodiimide method (Taylor & Conrad, 1972), prior to methylation analysis.

[c] Based on derived O-methylalditol acetates.

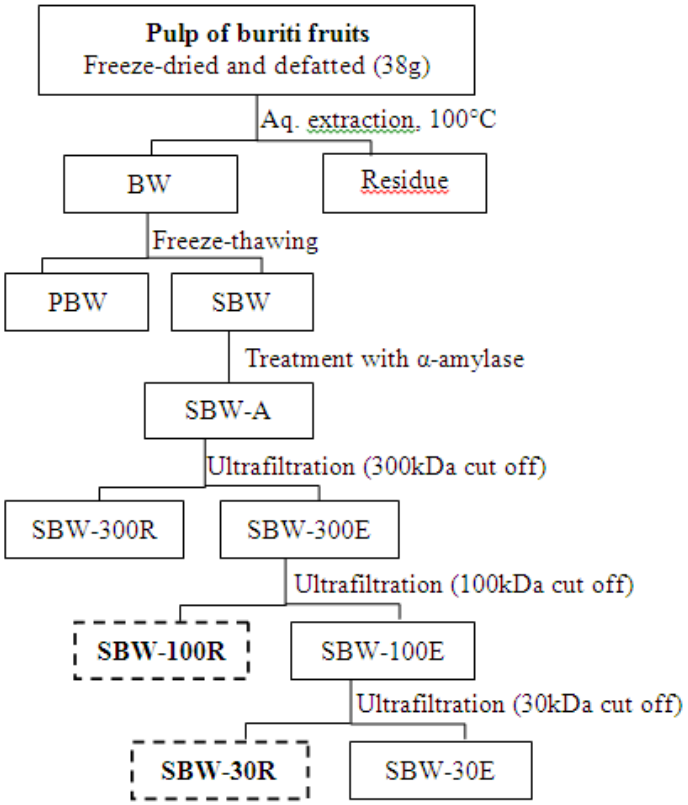

Figure 1. Scheme of extraction and fractionation of polysaccharides from the pulp of buriti fruits (*M. flexuosa*).

E= Eluted; R = retained in the ultrafiltration membrane.

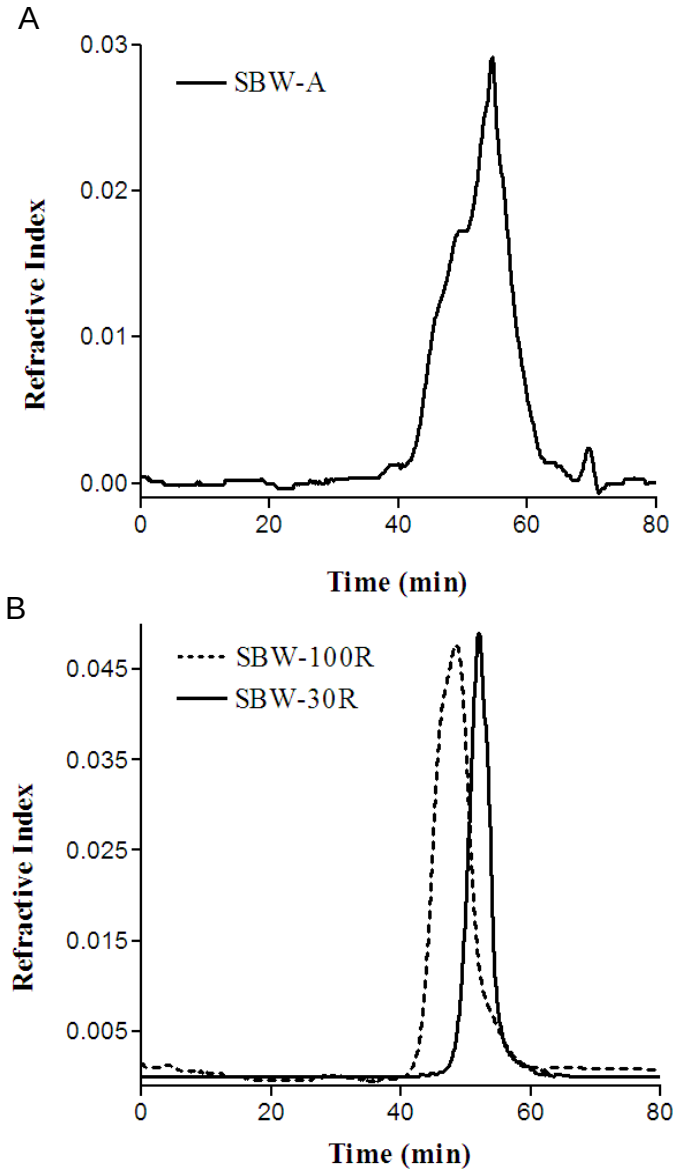

Figure 2. HPSEC elution profile of fraction SBW-A (A) and fractions SBW-30R and SBW-100R (B), obtained from buriti pulp (refractive index detector).

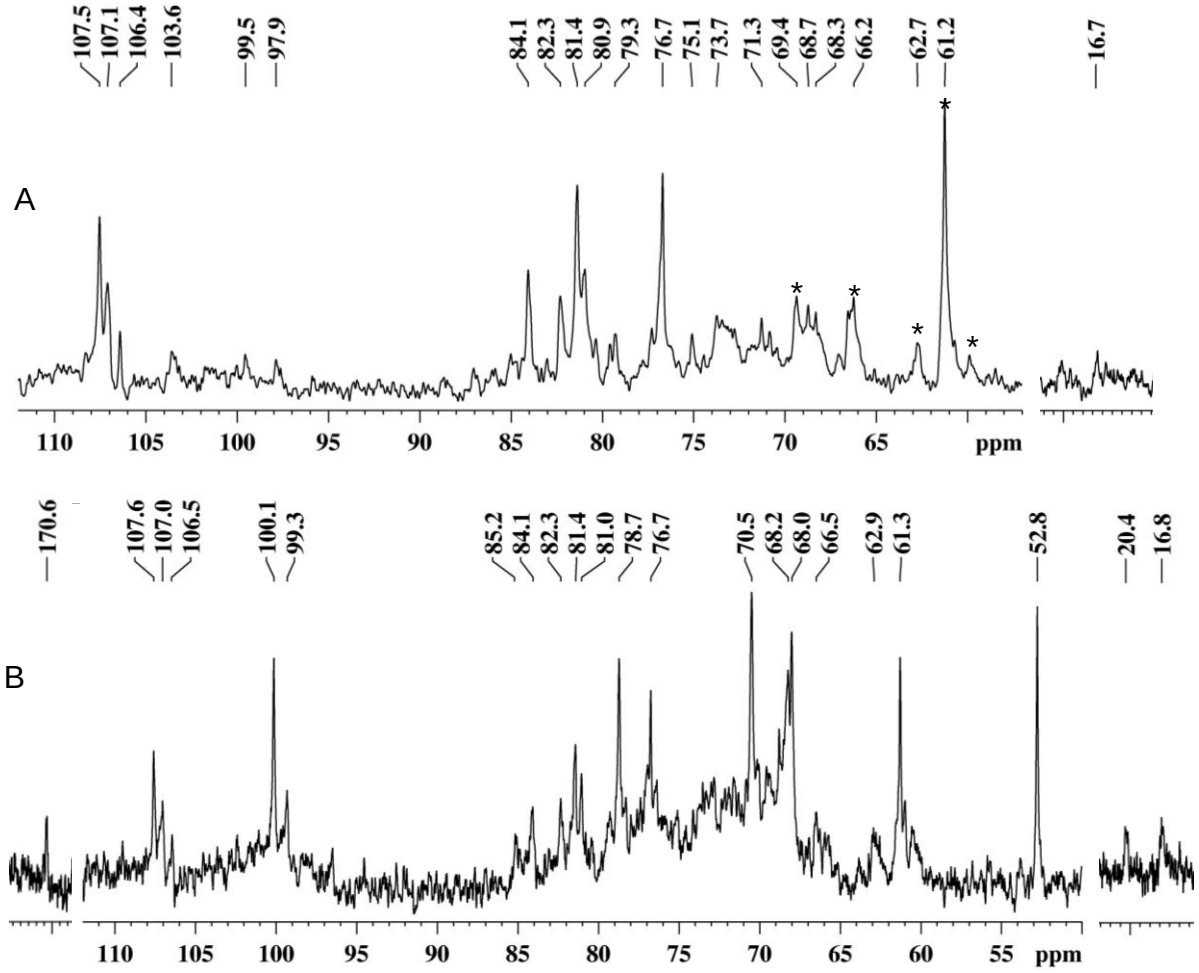

Figure 3. ^{13}C-NMR spectra of (A) fraction SBW-100R and, (B) fraction SBW-30R, in D_2O at 50ºC, (chemical shifts are expressed as δ PPM) obtained from buriti pulp. Inverted signals in DEPT-135 experiment are marked with asterisk.

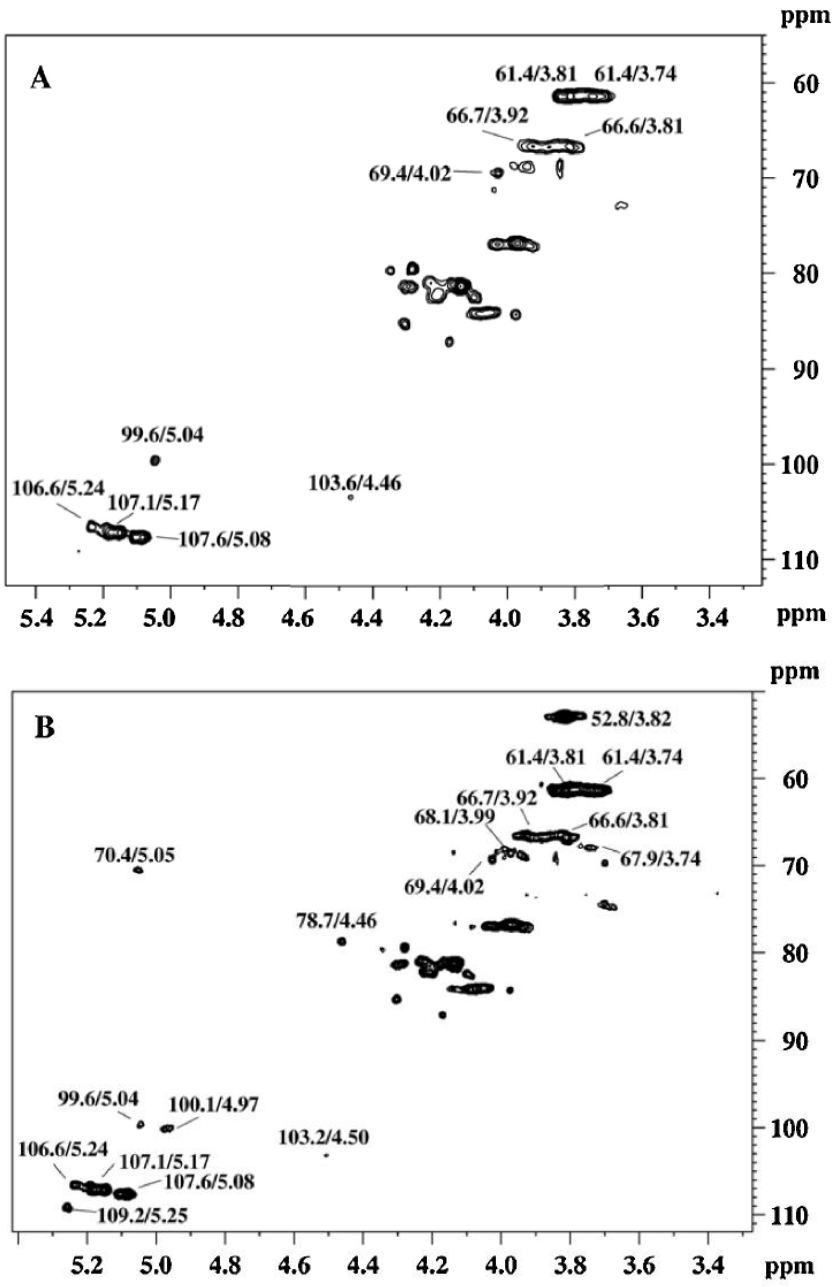

Figure 4. 2D ^{1}H–^{13}C HSQC correlation map showing some carbon and hydrogen assignments (discussed in the text) from (A) fraction SBW-100R and (B) fraction SBW-30R obtained from buriti pulp. Samples were dissolved in D_2O and data collected at probe temperature of 50 ^{o}C.

ARTIGO II

(Submetido para Plant Physiology and Biochemistry)

Structural diversity of hemicelluloses in tucumã (*Astrocaryum aculeatum)* primary cell walls, a commelinid monocotyledon from Arecaceae family

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ABSTRACT

Primary cell walls of members of Arecaceae family in the commelinid clade of monocotyledonous plants were previously suggested to have pectic polymers as main constituents, instead of hemicellulosic glucuronoarabinoxylans. However, few studies have been conducted to evaluate this assumption. We aimed to extract and characterize the main polysaccharides in tucumã (*Astrocaryum aculeatum*) primary cell walls, a member of Arecaceae family. Hemicellulosic polysaccharides (alkali extracted) in tucumã fruits were present in a greater proportion (22.3%) than pectic (water extracted) ones (10.6%). Thus, the formers were analyzed using monosaccharide composition, methylation, molecular weight determination and ^{13}C-NMR data. In sum, a highly ramified acidic galactoarabinoxylan (11.7%), a linear (1→5)-linked α-L-arabinan (6%), a low branched glucuronoxylan (3.1%) and small portions of a xyloglucan (0.9%) have been found in tucumã alkaline extract. These results are contrary to the previous hypothesis for Arecaceae primary cell wall structures and indicate that polysaccharides in tucumã are mainly hemicellulosic. Specific structural features are discussed by the taxonomic point of view.

Keywords: commelinid Arecaceae, primary cell walls, polysaccharides, tucumã, *Astrocaryum aculeatum*, palms.

1. Introduction

The diversity of plant cell walls polysaccharides greatly varies according to the type of tissue, plant developmental stage and taxa. The non-lignified cell walls observed in growing tissues (primary cell walls) possess distinct features from that of lignified secondary cell walls, present in vascular and woody tissues (DOMOZYCH, 2015). Regarding taxa variations, dicotyledonous plants in general, are known to possess large amounts of pectic polysaccharides and smaller amounts of hemicellulosic polymers (xyloglucans > heteroxylans, glucomannans and/or galactoglucomannans). On the other hand, monocotyledonous plants seem to have greater variations in cell wall compositions depending on the clade and/or order to which the plant belongs (HARRIS, 2005).

Based on the presence or absence of ester-linked ferulic in plant cell walls, Harris and Hartley (1980) classified monocots into two clades: commelinoids and non-commelinoids (further named commelinids/non-commelinids) (The Angiosperm Phylogeny Group, 2003). The ferulic acid absent, non-commelinid clade, embraces the Asparagales, Liliales, Pandanales, Dioscoreales, Petrosaviales, Alismatales, and Acorales orders (BREMER et al., 2009; GIVNISH et al., 2010). In contrast, the ferulic acid present commelinid clade includes Poales, Zingiberales, Commelinales, Dasypogonaceae and Arecales (CHASE et al., 2006; GIVNISH et al., 2010). The primary cell walls in non-commelinid clade were shown to possess high proportions of pectic polysaccharides and smaller portions of hemicelluloses (xyloglucans > heteroxylans), similar to what is found in dicotyledonous plants. In commelinid plants however, variations within families may occur. In general, primary cell wall composition in all families, except Arecaceae (Arecales order), possess high proportions of the hemicellulosic polymer glucuronoarabinoxylan, in detriment of pectic polymers and xyloglucans. The terminal family in the phylogenetic tree, Poaceae (grasses) from Poales order, also presents (1→3,1→4)-β-glucans as a major polymer. It was hypothesized that Arecaceae members (palms) however, a basal family in the commelinid clade, possess cell wall features similar to those from the non-commelinid clade and dicotyledonous plants which are rich in pectins, with smaller amounts of hemicellulosic polymers (HARRIS, 2005; HARRIS et al., 1997).

Notably, the latter assumptions were drawn based on monosaccharide surveys only, from few Arecaceae species.

Many of the early studies of wall compositions are also difficult to interpret and use in comparisons among taxa because the wall preparations were from whole organs, or even whole plants, and contained a mixture of wall types (DARVILL et al., 1980). In this sense, cells present in fruit pulp are generally thought to contain only primary cell walls (JOHN; DEY, 1986), and thus, their chemical structure evaluation can be used as a tool to better understand the primary cell wall composition of palms. So far, studies conducted to evaluate cell wall compositions from palm fruits are sparse and the results insufficient to ascertain a pattern in cell wall compositions in Arecaceae members (CANTU-JUNGLES et al., 2015; CORDEIRO et al., 2015; ISHURD et al., 2002; SILVA; PARENTE, 2001).

Thus, the pulp of tucumã fruits (*Astrocaryum aculeatum*), a palm plant native from non-flooded regions in the Amazon forest, was used to evaluate its primary cell wall structure and hemicellulosic diversity. Furthermore, this paper brings new insights into the polysaccharide composition of Arecaceae commelinid monocotyledons.

2. Materials and Methods

2.1 Plant material

Ripe tucumã fruits (*A. aculeatum)* were purchased at local market in Manaus city, State of Amazonas, Brazil.

2.2 Extraction and purification of polysaccharides

The fruits were peeled and the pulp was manually removed (600 g), freeze-dried and milled (328.2 g). Dried pulp powder was defatted with chloroform-methanol (1:1) in a Soxhlet apparatus to remove lipids, pigments and other hydrophobic materials. The polysaccharides were exhaustively extracted from the residue (94 g) with water under reflux at 100 ºC for 2 h (1 L each). Aqueous extracts were obtained by centrifugation (8000 rpm/ 15 ^{o}C/ 1 5 min), joined and concentrated under reduced pressure. The polysaccharides were precipitated with EtOH (3 vol.), collected by centrifugation (8000 rpm/ 15 ^{o}C/ 15 min) and freeze-dried, giving fraction TW. The residue from aqueous extraction was submitted to alkaline extraction with aq. 10% NaOH at 100 ºC for 2 h (3x, 1 L each). Alkaline extracts were obtained by centrifugation (8000 rpm/ 15 ^{o}C/ 15 min), joined, neutralized, dialyzed, concentrated under reduced pressure and freeze-dried, giving fraction TK.

A freeze-thaw treatment was applied in fraction TK, to give cold-water soluble (STK) and insoluble fractions (PTK). In this procedure, the sample was frozen and then thaw at room temperature followed by centrifugation (8000 rpm/ 15 ^{o}C/ 15 min) (Fig. 1). Fraction STK was further treated with Fehling solution as previously described (JONES; STOODLEY, 1965) and the precipitated materials separated from the soluble materials by centrifugation. The Cu^{2+}-precipitate (fraction PF-STK) and supernatants (fraction SF-STK) were neutralized with AcOH, dialyzed against tap water, deionized with cationic resin and then freeze–dried.

Fraction PTK was treated with 300µl of α-amylase (from *Bacillus licheniformis*, Sigma A3403) at 37 ^{o}C overnight and further dissolved in 20 ml of alkaline Fehling's solution (250 g/L KOH, 346 g/L sodium potassium tartarate). After dissolution, an equal volume of cupric Fehling's solution (111.5 g/L $CuSO_4 \cdot 5H_2O$) was added, and the precipitated material recovered by centrifugation (3860*g*, 20 min at 4 ºC). The supernatant (SF-PTK) and precipitated (PF-PTK) fractions were neutralized with AcOH, dialyzed against tap H_2O, deionized with cationic resin and then freeze–dried.

Yields of polysaccharide fractions were expressed as a percentage from the weight of defatted dried pulp of tucumã that was submitted to extraction (94 g), whereas the moisture and lipids contents were expressed as percent based on the weight of the tucumã fruit wet pulp (600 g).

2.3 Sugar composition

Neutral monosaccharide components of the polysaccharides were determined after hydrolysis with 2 M TFA (8 h/ 100 °C), followed by conversion to alditol acetates with successive $NaBH_4$ reduction, and acetylation with Ac_2O-pyridine (1:1, v/v, 1ml) at 100 °C for 30 min. These were analyzed through GC–MS using a Varian gas chromatograph and mass spectrometer, model Saturn 2000R, with He as carrier gas. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 220 ºC and held at this constant temperature for 19.75 min was used for the quantitative analysis.

Uronic acid contents were determined spectrophotometrically using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

2.4 Determination of homogeneity and average molecular weight

The soluble polysaccharides homogeneity and average molecular weight were evaluated by high performance steric exclusion chromatography (HPSEC), with a Waters 2410 differential refractometer as equipment for detection. A series of four columns, with exclusion sizes of 7×10^6 Da (Ultrahydrogel 2000, Waters), 4×10^5 Da (Ultrahydrogel 500, Waters), 8×10^4 Da (Ultrahydrogel 250, Waters) and 5×10^3 Da (Ultrahydrogel 120, Waters) was used. The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. The sample, previously filtered through a membrane (0.22 µm, Millipore), was injected (250 µl loop) at a concentration of 1 mg/ml, at room temperature. The specific refractive index increment (*dn/dc*) was determined and the results were processed with ASTRA software provided by the manufacturer (Wyatt Technologies).

2.5 Methylation analysis

Fraction SF-STK was *O*-methylated according to the method of Ciucanu and Kerek (1984). The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 8 h) and neutralization with $BaCO_3$. The resulting material was submitted to reduction and acetylation as described above for sugar composition, except that the reduction was performed using $NaBD_4$. The products (partially *O*-methylated alditol acetates) were examined by capillary GC-MS. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 210 ºC and held at this temperature for 31 min was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (SASSAKI et al., 2005a).

2.6 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C-NMR spectra were obtained with a Bruker DRX 400 MHz AVANCE III NMR spectrometer (Bruker Daltonics, Germany), operating at 9.5 T, observing ^{13}C at 100.61 MHz. Analyses were performed with a 5 mm inverse gradient probe, at 70 ºC, the water soluble samples being dissolved in D_2O and the water-insoluble ones in DMSO-d_6. Chemical shifts are expressed as δ PPM, using the resonances of CH_3 groups of acetone internal standard (δ 30.2), or DMSO-d_6 (δ 39.7). All pulse programs were supplied by Bruker.

3. Results

The fresh pulp of tucumã fruits (600 g) was freeze-dried, yielding moisture of approximately 45%. The dried pulp powder (328.2 g) was then defatted with

chloroform-methanol (1:1) in a Soxhlet apparatus, yielding approximately 39% of nonpolar compounds in tucumã pulp. The defatted residue (94 g) was then submitted to successive extraction with water at 100 ºC, and the extracted polysaccharides (fraction TW, 10.6% yield) recovered by EtOH precipitation and dialysis (Fig. 1). The residue from aqueous extraction was further submitted to alkaline extraction at 100 ºC, yielding fraction TK (22.3%).

The majority of pectic polymers are prone to be extracted through aqueous extraction. Hemicelluloses however, are found more closely attached to cellulose in the plant cell walls structure and thus, although present in some water extracts, are usually get in higher yields using alkaline solutions (ASPINALL, 1973). Thus, the higher yields of alkaline extract (TK) than water extract (TW) indicates that tucumã cell wall polysaccharides are mainly hemicellulosic. Thus, fraction TK was chosen for further purification and characterization. As the first step, fraction TK was submitted to freeze-thawing treatment, giving rise to cold-water soluble (STK, 13% yield) and cold-water insoluble (PTK, 8.8% yield) fractions.

According to monosaccharide composition data (Table 1), fraction STK was composed mainly of arabinose (66.9%) and xylose (19.3%). It's known that when treated with Fehling's solution, linear xylans form a complex with copper and precipitate (CARBONERO et al., 2002). Thus, as an attempt to precipitate the xylose containing polymer present in fraction STK, it was submitted to Fehling*'s* treatment, yielding Fehling supernatant (SF-STK, 11.7% yield) and precipitated (PF-STK, 0.9% yield) fractions (Fig. 1). When analyzed through HPSEC, both fractions SF-STK and PF-STK presented homogeneous elution profiles, with M_w of 24 and 119 kDa, respectively (Figure 2).

Fraction SF-STK presented arabinose: xylose: uronic acids: galactose in a 6.5:5.8:1.2:1.0 ratio in monosaccharide composition analysis and indicated that an acid arabinoxylan is the main component in this fraction (Table 1). To further elucidate its linkage pattern, methylation analysis was conducted in SF-STK (Table 2). The presence of low proportions of the derivative 2,3-Me_2-Xyl-ol acetate (7.1%), relative to the 4-*O*-linked xylan backbone and high proportions of 2-Me-Xyl-ol acetate (17.6%) and 3-Me-Xyl-ol acetate (14.5%), indicate that the arabinoxylan is highly substituted with side chains attached at *O*-3 and *O*-2 positions, respectively. The

arabinose derivatives 2,3,5-Me_3-Ara-ol acetate (18.9%), 3,5-Me_2-Ara-ol acetate (11.4%) and 2,3-Me_2-Ara-ol acetate (20.8%) indicate that side chains are present as terminal, 2-*O*- and 5-*O*-linked arabinose units. The derivative 2,3,4,6-Me_4-Gal-ol acetate (7.3%) indicate that terminal galactose is also present as a side chain. Moreover, uronic acids (8.3%) were observed in monosaccharide composition of SF-STK (Table 1). Probably, these are due to the presence of glucuronic acid, commonly found in arabinoxylans from other monocotyledonous plants (HARRIS, 2005). Thus, an acid galactoarabinoxylan was the polymer present in SF-STK. These data were corroborated by ^{13}C-NMR analysis (Fig. 3A), which presented a spectrum similar to other arabinoxylans (PRASHANTH; MURALIKRISHNA, 2014; SAGHIR et al., 2008; SUN et al., 2011). Anomeric signals of α-L-Ara*f* units can be observed at δ 107.5, while those of β-D-Xyl*p* units appeared at δ 101.2/101.7 (PRASHANTH; MURALIKRISHNA, 2014; SAGHIR et al., 2008; SUN et al., 2011). Although xylans are expected to be precipitated by Fehling's solution, the large proportions of arabinose side chains linked to the xylan backbone prevented its backbone from interact ing with Cu^{2+}, and thus the arabinoxylan remained soluble and was present in fraction SF-STK.

Fraction PF-STK presented glucose and xylose as main sugars, and minor amounts of galactose and fucose in the monosaccharide composition analysis (Table 1). These could be indicative of the presence of a fucogalactoxyloglucan in fraction PF-STK, which was corroborated by the ^{13}C-NMR analysis (Fig. 3B). The anomeric signal related to (1→4) and (1,4→6)-linked β-Glc*p* units that form the main chain of the xyloglucan is found at δ 102.3 (BUSATO; VARGAS-RECHIA; REICHER, 2001; HANTUS et al., 1997). The signal at δ 98.9 could be assigned either to anomeric carbons of terminal and/or 2-*O*-substituted α-Xyl*p* units, while the signals at δ 103.2 and δ 99.5 were assigned to C-1 of β-Gal*p* and α-Fuc*p* units, respectively (BUSATO et al., 2001). Once PF-STK represented a minor polysaccharide fraction in tucumã pulp (0.9% yield), methylation analysis has not been carried out.

Regarding cold-water insoluble polysaccharides (fraction PTK), monosaccharide analysis showed arabinose (37.6%), xylose (36.1%) and glucose (24.4%) as main sugars (Table 1). Moreover, its ^{13}C-NMR spectrum (data not shown) presented five intense signals of β-Xyl*p* at *δ* 101.8 (C-1), 75.6 (*O*-substituted C-4),

74.0 (C-3), 72.1 (C-2) and 63.2 (C-5), characteristic of (1→4)-β-D-Xyl*p* units (CORDEIRO et al., 2015; KOVAČ et al., 1980). Moreover, signals from α-L-Ara*f* units could be observed at *δ* 108.1 (C-1), 82.1 (C-4), 81.7 (C-2), 77.7 (C-3) and 67.2 (*O*-substituted C-5), indicating the presence of (1→5)-α-L-Ara*f* units (CORDEIRO et al., 2015; CORDEIRO et al., 2012). Finally, the presence of glucose in monosaccharide composition together with ^{13}C-NMR signals from (1→4)-α-D-Glc*p* at *δ* 100.0 (C-1), 72.1 (C-2), 73.2 (C-3), 78.9 (C-4), 71.7 (C-5) and 60.7 (C-6), indicate the presence of starch in fraction PTK (NASCIMENTO et al., 2013b).

To remove starch and further purify polymers in fraction PTK, an α-amylase digestion followed by Fehling treatment was performed, yielding fractions SF-PTK (supernatant from Fehling treatment, 6% yield) and PF-PTK (precipitate from Fehling treatment, 3.1% yield) (Fig. 1). This treatment was highly efficient to separate distinct polymers once fraction SF-PTK presented only arabinose on its monosaccharide composition (Table 1), and its ^{13}C-NMR spectrum contained only the five main signals of (1→5)-α-L-Ara*f* units (Fig. 4A), indicating the presence of a linear arabinan in SF-PTK. On the other hand, fraction PF-PTK presented xylose (90.1%) as main monosaccharide, together with small amounts of glucose (7.0%) and uronic acids (2.9%). Its ^{13}C-NMR spectrum (Fig. 4B) showed the five main signals described above for (1→4)-β-D-Xyl*p* units. Moreover, the small anomeric signal at *δ* 97.5 was assigned to α-D-glucuronic acid (NASCIMENTO et al., 2013b), also found in 2.9% on its monosaccharide composition (Table 1). These data indicate that while the arabinan remained soluble in Fehling solution (fraction SF-PTK), a glucuronoxylan was precipitated in fraction PF-PTK. Lastly, the presence of glucose in monosaccharide composition (Table 1), together with minor ^{13}C-NMR signals from (1→4)-α-D-Glc*p* units (*δ* 100.0, 72.0, 73.2, 78.8, 71.6 and 60.6), indicate that the starch in PTK could not be completely removed with amylase treatment, and a small portion remained in fraction PF-PTK.

In sum, polysaccharides present tucumã (*A. aculeatum)* cell walls were mainly extracted through alkaline extraction. Arabinoxylan was present as 53.7% of alkali extracted polysaccharides, followed by arabinan (27.8%), glucuronoxylan (14.1%) and small portions of xyloglucan (4.4%).

4. Discussion

Members from Arecaceae, a basal family in the monocots commelinid clade, are believed to diverge from other commelinid and to contain a primary cell structure rich in pectic polymers, similar to that found in non-commelinid monocotyledonous and dicotyledonous plants (HARRIS, 2005; HARRIS et al., 1997). However, our results indicate that hemicellulosic polymers were the main components in tucumã primary cell walls.

The hemicellulosic polysaccharide found in larger amount in tucumã alkaline extract was characterized as a highly branched acid galactoarabinoxylan. Notably, arabinoxylans are usually found in the commelinid clade of monocotyledonous plants constituting about 20% of the primary cell walls (SCHELLER; ULVSKOV, 2010). In the Arecaceae family however, based on analysis of primary cell walls of the palms *Phoenix canariensis* and *Rhopalostylis sapida*, Carnachan and Harris (2000) have proposed the occurrence of very low amounts of arabinoxylans. These polymers are also rarely reported in dicotyledonous plants, such as *Listea* (DAS et al., 2013; HERATH; KUMAR; WIMALASIRI, 1990; WIMALASIRI; KUMAR, 1995) and tomato mucilage (*Solanum lycopersicum*) (NASCIMENTO et al., 2016). Thus, the presence of high amounts of arabinoxylan in an Arecaceae family member, as reported herein for tucumã, and similar to that is observed in other commelinid monocotyledonous plants, represents a new finding with taxonomic relevance.

The second most abundant hemicellulose in tucumã alkaline extract was a linear (1→5)-α-L-arabinan. The presence of this kind of hemicellulosic polymer in Arecaceae has been previously reported only for buriti (*Mauritia flexuosa)*, and is scarcely observed even in the cell walls of other monocotyledonous and dicotyledonous plants (CORDEIRO et al., 2015). Although Carnachan and Harris (2000) have found arabinan in palms such as *P. canariensis* and *R. sapida*, these were mostly found associated with pectin. Finally, glucuronoxylans and very low amounts of xyloglucans were also present in TK. Peña et al. (2016) analyzed xylans present in 29 monocotyledonous species and found the presence of glucuronoxylans, without any attached Ara*f*/Ara*p* residue, only in species from the

non-commelinid clade and the palms *Cocos nucifera* and *Howea forsteriana* from Arecaceae family.

Overall, our results indicated that polysaccharides in tucumã primary cell walls are mainly hemicellulosic, and have characteristics of both, commelinid and non-commelinid monocotyledons, together with the rarely found linear (1→5)-α-L-arabinan. That is contrary to what was previously hypothesized for Arecaceae family members (CARNACHAN; HARRIS, 2000; HARRIS et al., 1997). Likewise, studies conducted in our lab to evaluate polysaccharides present in açaí berries, the fruits of the palm *Euterpe oleraceae*, indicated that their major polysaccharides are hemicellulosic (linear (1→4)-β-D-xylan), with a lesser percentage of pectic polymers (unpublished data). In buriti fruits (*M. flexuosa*) however, the cell wall polysaccharides were arabinan-rich pectins (CANTU-JUNGLES et al., 2015), although the presence of linear (1→5)-α-arabinan, (1→4)-β-xylan and (1→3,1→4)-α-glucan were observed as well, in almost equal amounts (CORDEIRO et al., 2015). Taken together, these data indicate that the cell walls of Arecaceae members possess a greater diversity than previously hypothesized (HARRIS, 2005), and depending on the genera may present characteristics more similar to commelinid and/or non-commelinid clades of monocotyledonous plants. Further studies on polysaccharides of Arecaceae members should be encouraged aiming the examination of the extent of their cell walls diversity.

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Table 1 - Monosaccharide composition of fractions obtained from alkaline extraction of tucumã (*A. aculeatum*).

Fraction	Neutral sugars[a]					Uronic acid[b]
	Fuc	Ara	Xyl	Gal	Glc	
STK	3.4	66.9	19.3	3.5	6.9	n.d.[c]
PTK	-	37.6	36.1	1.9	24.4	n.d.
SF-STK	tr [d]	45.1	39.7	6.9	tr	8.3
PF-STK	6.2	tr	19.4	12	62.4	tr
SF-PTK	-	100	-	-	-	-
PF-PTK	-	tr	90.1	tr	7	2.9

[a] % of peak area relative to total peak areas, determined by GC-MS.

[b] Determined using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

[c] Not determined

[d] Trace amounts (<1%).

Table 2 - Linkage types based on analysis of partially *O*-methylalditol acetates obtained from the methylated fraction SF-STK from tucumã (*A. aculeatum*).

Partially *O*-methylalditol acetate	SF-STK[b]	Linkage type[c]
2,3,4-Me_3-Xyl[a]	2.4	Xyl*p*-(1→
2,3- Me_2-Xyl	7.1	→4)- Xyl*p*-(1→
2–Me-Xyl	17.6	→3,4)- Xyl*p*-(1→
3–Me-Xyl	14.5	→2,4)- Xyl*p*-(1→
2,3,5-Me_3-Ara	18.9	Ara*f*-(1→
3,5-Me_2-Ara	11.4	→2)-Ara*f*-(1→
2,3-Me_2-Ara	20.8	→5)-Ara*f*-(1→
2,3,4,6-Me_4-Gal	7.3	Gal*p*-(1→

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-Methylarabinitolacetate, etc.

[b] % of peak area of *O*-methylalditol acetates relative to total area, determined by GC-MS.

[c] Based on derived *O*-methylalditol acetates.

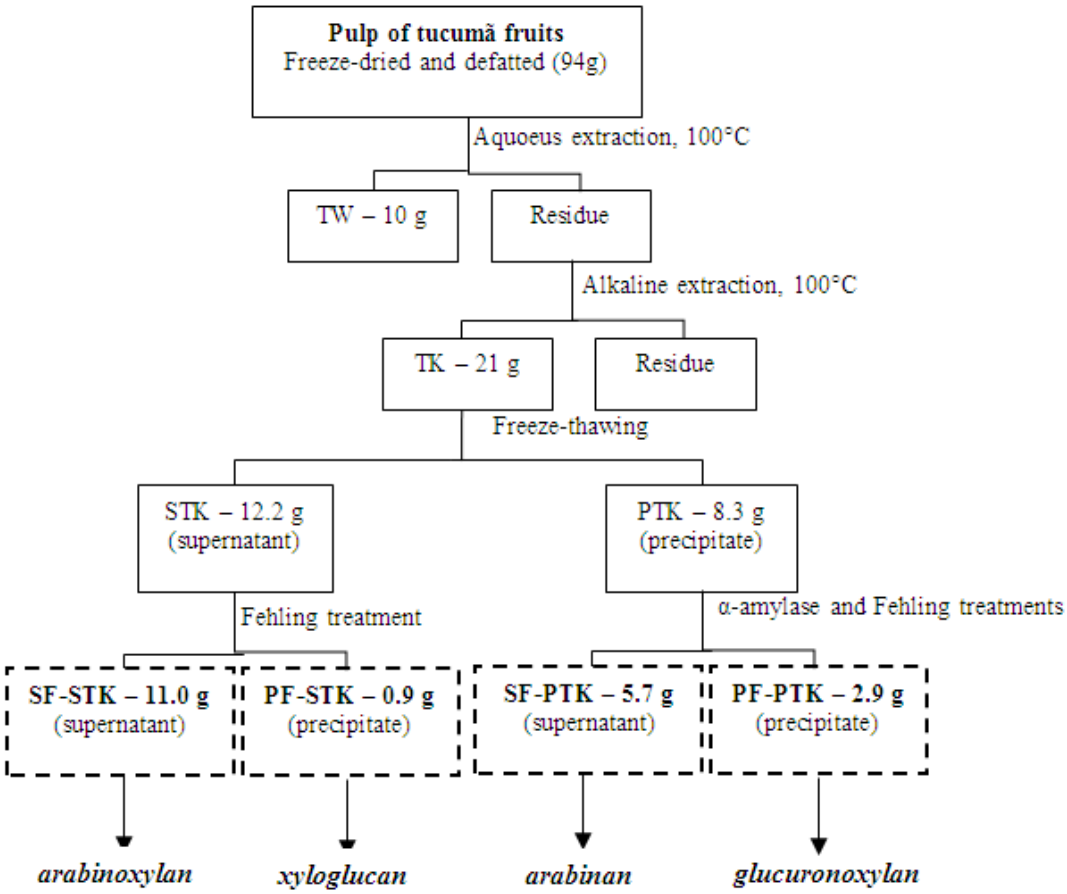

Figure 1. Scheme of extraction and fractionation of polysaccharides from tucumã pulp (*A. aculeatum).*

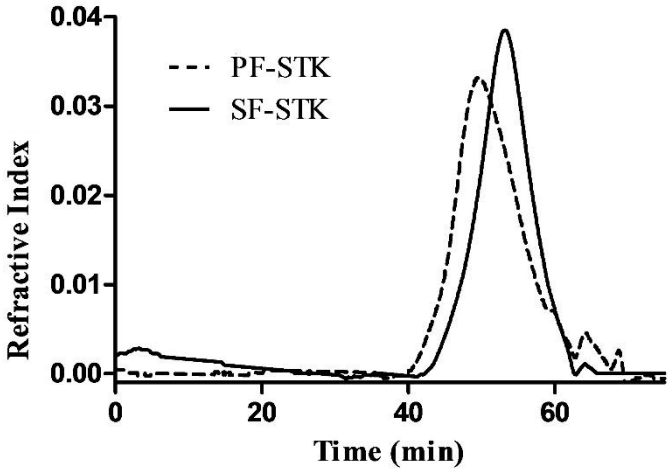

Figure 2. HPSEC elution profile of fractions SF-STK and PF-STK obtained from tucumã pulp (refractive index detector).

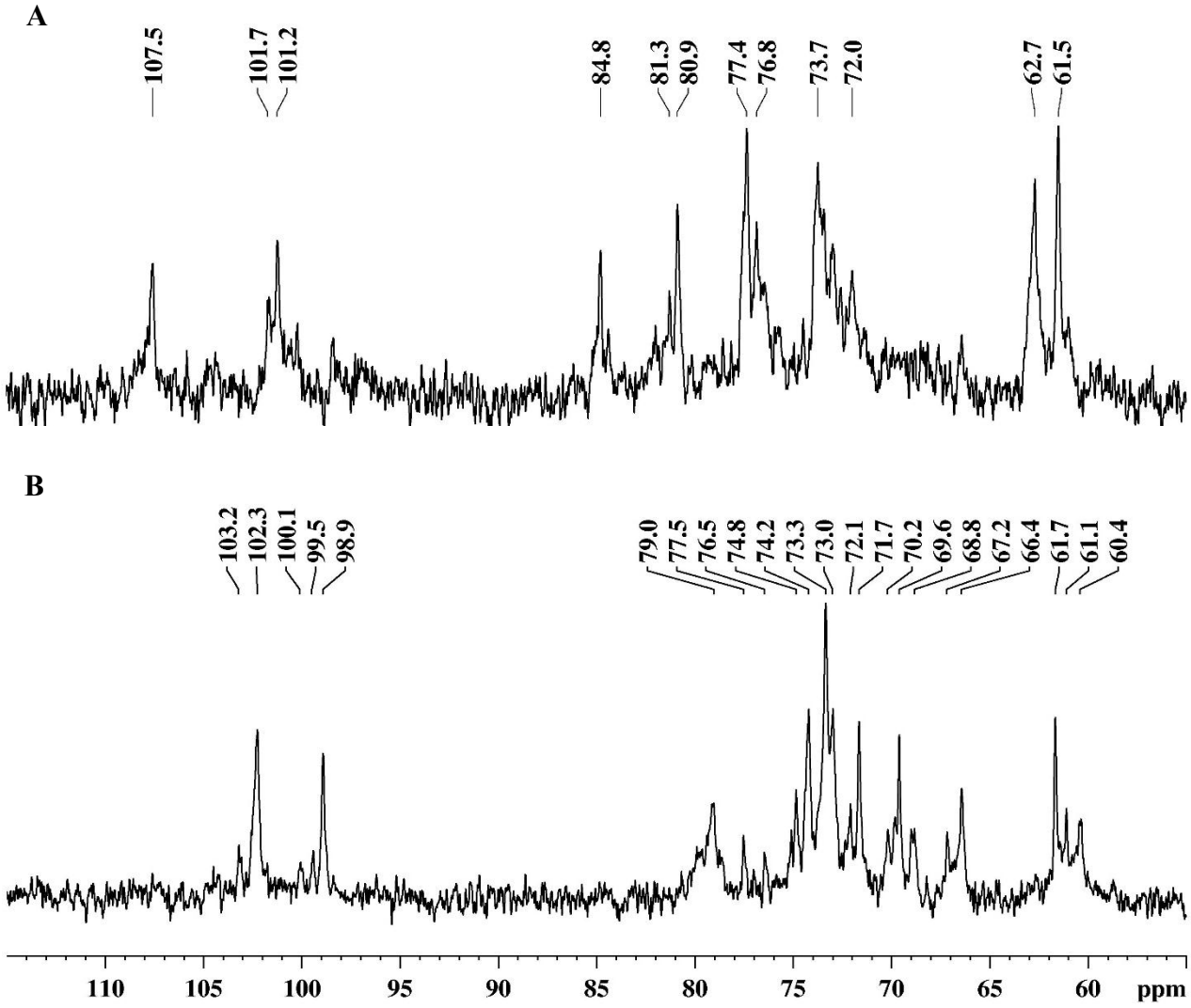

Figure 3. ^{13}C-NMR spectra of fractions SF-STK (A) and PF-STK (B) in D_2O at 70 ºC.

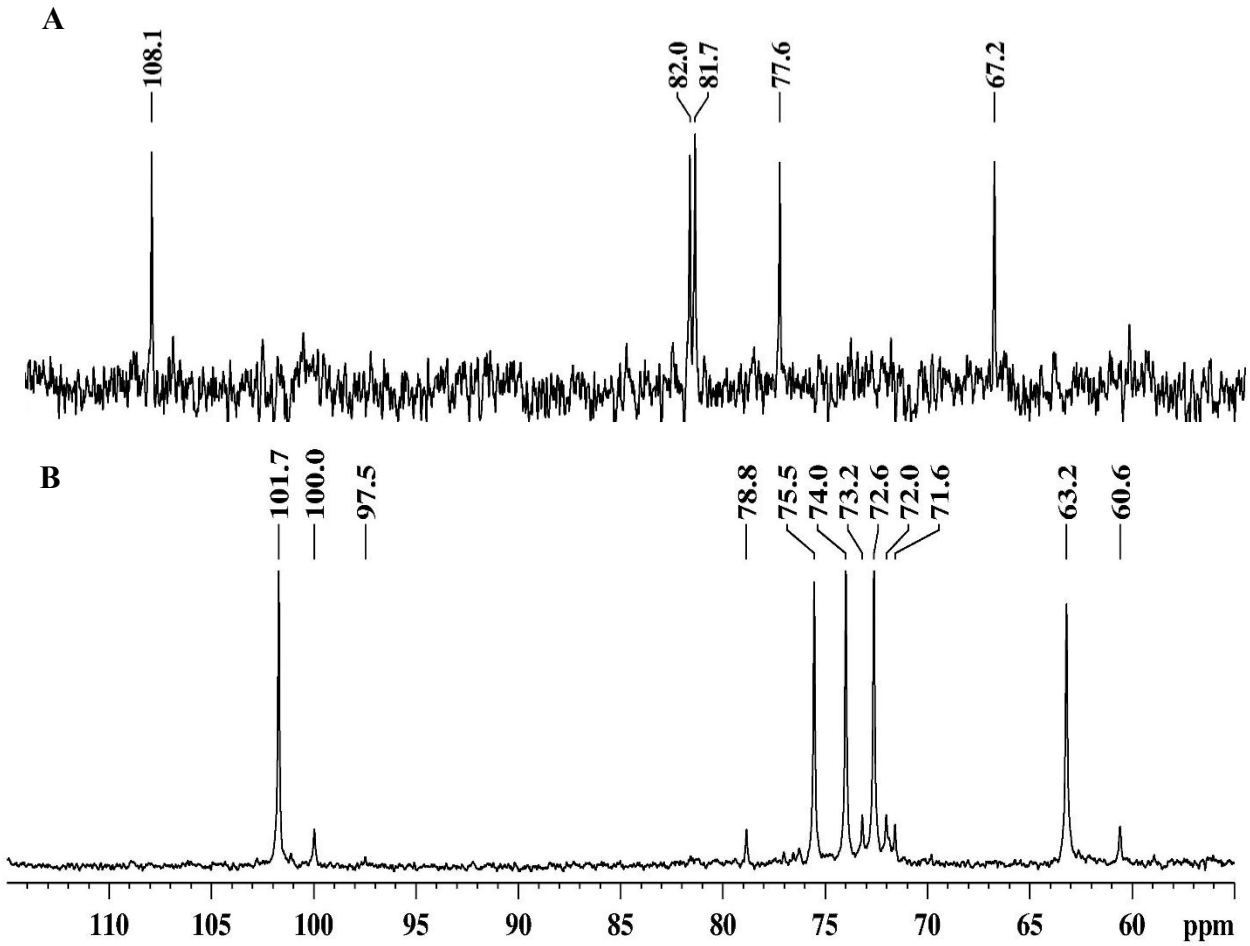

Figure 4. ^{13}C-NMR spectra of fractions SF-PTK (A) and PF-PTK (B) in DMSO-d_6 at 70 ºC.

ARTIGO III

(Submetido para Carbohydrate Polymers - em fase de revisão)

Pectins from primary cell walls of edible açaí (*Euterpe oleraceae*) berries, fruits of a monocotyledon palm

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ABSTRACT

Açaí berries (*Euterpe oleracea*) are greatly consumed in Brazil and exported to other countries as a nutritional supplement, due to health benefits attributed to its consumption. However, the complete chemical structure of bioactive polysaccharides was not fully elucidated yet. In this work, we characterize pectic polysaccharides from açaí berries through monosaccharide composition, HPSEC, methylation and ^{13}C and $^{1}H/^{13}C$ HSQC-DEPT-NMR analyses. A highly methoxylated homogalacturonan with a DM of 88% and M_w of 22 kDa together with small amounts of a mannoglucan were found. Moreover, a type II arabinogalactan (M_w = 45 kDa) containing a backbone with high portions of 6-*O*-linked and 3,6-*O*-linked Gal*p* chains rather than 3-*O*-linked Gal*p* was also isolated and structurally characterized. These findings contribute to correlate the fine chemical structure with the previously reported action of açaí polysaccharides on innate immune response. Moreover, from the taxonomic point of view, the results bring new information about polysaccharide composition of primary cell walls of palms (Arecaceae), that despite being commelinid monocots, have a distinct cell wall composition.

Keywords: açaí berries; palm fruits; type II arabinogalactan; highly methoxylated homogalacturonan; Arecaceae.

1. Introduction

Euterpe oleracea Mart. (Arecaceae) is a palm plant widely spread in Northern South America with its greatest abundance in the Amazonian flood plains of the Amazon River in Brazil. Its fruits, commonly known as açaí, are small dark black-purple rounded in shape and possess a small edible layer that uncovers their big seeds which are responsible for most of the fruit size (QUEIROZ; JÚNIOR, 2001; YAMAGUCHI et al., 2015). For separating the seeds from the pulp, the fruits are usually macerated with water and a thick, purple-colored beverage is obtained ('açaí pulp'). The açaí pulp is consumed as such or further used in different kinds of beverages and desserts (BRONDIZIO, 2008; YAMAGUCHI et al., 2015).

In 2014, it was estimated that 198,149 ton of açaí were extracted in Brazil and around 5,000 ton were exported generating US$ 22.523 million in exportation to countries such as United States and Japan (CONAB, 2016). In the last decades it has also gained attention as a nutritional supplement, being considered a major botanical dietary supplement in United States (NBJ'S SUPPLEMENT BUSINESS REPORT, 2011).

The açaí berries hold many potential health benefits that are being studied. Among the biological evaluations, antioxidant and immunomodulatory effects have been most frequently investigated, but the responsible constituents have not been fully resolved (YAMAGUCHI et al., 2015). Besides the high amounts of flavonoids, açaí berries are also rich in carbohydrates, that compose around 42% of the fruit total weight in a dry basis (ODENDAAL et al., 2014; MENEZES; TORRES; SRUR, 2008). Holderness et al. (2011), investigated immune responses of polyphenols and polysaccharides extracted from açaí and found that a polysaccharide fraction containing a type II arabinogalactan was responsible, at least in part, for the immune responses reported, rather than polyphenols. However, isolation and fine chemical structure of polysaccharides from açaí have not been reported.

Based on monosaccharides analysis surveys, it has been observed that members of Arecaceae family have large proportions of pectic polysaccharides and

smaller amounts of xyloglucans/heteroxylans, differently to what occur in the other members of commelinid monocots, which have mainly glucuronoarabinoxylans and (1→3)(1→4)-β-glucans in their cell walls (CARNACHAN; HARRIS, 2000; HARRIS, 2005). However, studies investigating the detailed chemical structure of cell walls from palm tree fruits were only found for dates (*Phoenix dactylifera* L.;) (ISHURD; KENNEDY, 2005), *Orbignya phalerata* fruits (SILVA; PARENTE, 2001) and buriti (*Mauritia flexuosa*) (CANTU-JUNGLES et al., 2015; CORDEIRO et al., 2015). Thus, further studies on cell wall polysaccharides from Arecaceae members are needed to corroborate the hypothesis that despite being commelinid monocots, they have a distinct cell wall composition, similar to that of dicotyledons and non-commelinid monocotyledons.

Once polysaccharides from açaí were shown to enhance immune function, and the chemical structure of carbohydrates is closely related to biological functions, the aim of this work was to provide a detailed chemical characterization of pectic polysaccharides present in the aqueous extract of edible açaí berries. Moreover, from the taxonomic point of view, this work brings new insights into the structure of polysaccharides present in the cell walls of commelinid monocots palm fruits of Arecaceae family.

2. Materials and Methods

2.1 Plant material

Ripe fruits of açaí (*E. oleracea*) were purchased at local market in Belém city, State of Pará, Brazil.

2.2 Extraction and purification of polysaccharides

The pulp of açaí berries was manually separated from the seeds (228 g pulp yield), freeze-dried and milled (185 g). Dried pulp powder was defatted with chloroform-methanol (1:1), in order to remove lipids, pigments and other hydrophobic materials. The polysaccharides were exhaustively extracted from the residue (148 g) with water at 100 ºC for 2 h (1 L each). Aqueous extracts were obtained by centrifugation (8000 rpm/ 15 ^{o}C/ 15 min), joined and concentrated under reduced pressure. The polysaccharides were precipitated with EtOH (3 vol.), collected by centrifugation (8000 rpm/ 15 ^{o}C/ 15 min) and freeze-dried, giving fraction AW.

A freeze-thaw treatment was applied in fraction AW, to give cold-water soluble (SAW) and insoluble fractions (PAW). In this procedure, the sample was frozen and then thaw at room temperature followed by centrifugation (8000 rpm/ 15 ^{o}C/ 15 min) (Fig. 1).

Proteins present in fraction SAW were removed using trichloroacetic acid (TCA) precipitation (20% w/v) according to Oliveira, Marques & Azeredo (1999), followed by centrifugation (8000 rpm/ 15 ^{o}C/ 15 min), to collect the 20% TCA-soluble fraction, SSAW (Fig. 1).

The fraction SSAW was later purified by ultrafiltration through membranes with cut-off of 50 and 100 kDa (PLHK04710-Ultracel, Millipore), yielding an eluted fraction in the 50 kDa membrane (50E), and retained and eluted fractions in the 100 kDa membrane (100R and 100E, respectively) (Fig. 1).

The eluted fraction through 100 kDa ultrafiltration membrane (100R) was further treated with Fehling solution and the precipitated material (PF) separated from the soluble material (SF) by centrifugation (Fig. 1). The Cu^{2+}-precipitate and supernatant were neutralized with AcOH, dialyzed against tap water, deionized with cationic ion exchange resin and then freeze–dried.

Yields of polysaccharide fractions were expressed as percent based on the weight of dried pulp of açaí berries that was submitted to extraction (185 g), whereas the moisture and lipids content were expressed as percent based on the weight of the wet açaí fruit pulp (228 g).

2.3 Sugar composition

Neutral monosaccharide components of the polysaccharides were determined by hydrolysis with 2 M TFA (8 h/ 100 °C), followed by conversion to alditol acetates by successive $NaBH_4$ reduction, and acetylation with Ac_2O-pyridine (1:1, v/v, 1ml) at 100 °C for 30 min. These were analyzed by GC–MS using a Varian gas chromatograph and mass spectrometer, model Saturn 2000R, with He as carrier gas. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 220 ºC and held at this constant temperature for 19.75 min was used for the quantitative analysis.

Uronic acid contents were determined using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

2.4 Determination of homogeneity and molecular weight of polysaccharides

The homogeneity and average molecular weight (M_w) of soluble polysaccharides were determined by high performance steric exclusion chromatography (HPSEC), using a Waters 2410 differential refractometer and a Pharmacia LKB Uvicord VW 2251 ultraviolet detector at 280 nm (UV) as detection equipments. Four columns were used in series, with exclusion sizes of 7×10^6 Da (Ultrahydrogel 2000, Waters), 4×10^5 Da (Ultrahydrogel 500, Waters), 8×10^4 Da (Ultrahydrogel 250, Waters) and 5×10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. The sample, previously filtered through a membrane (0.22 µm, Millipore), was injected (250 µl loop) at a concentration of 1 mg/ml. The specific refractive index increment (*dn/dc*) was determined and the results processed with ASTRA software provided by the manufacturer (Wyatt Technologies).

2.5 Methylation analysis of polysaccharide

Fraction SF was *O*-methylated according to the method of Ciucanu and Kerek (1984). The per-*O*-methylated polysaccharide was then submitted to methanolysis in 3% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 16 h) and neutralization with $BaCO_3$. The material was then submitted to reduction and acetylation as described above for sugar composition, except that the reduction was performed using $NaBD_4$. The products (partially *O*-methylated alditol acetates) were examined by capillary GC-MS. A capillary column (30 m x 0.25 mm i.d.) of DB-225, held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 210 ºC and held at this temperature for 31 min was used for separation. The partially *O*-methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times (SASSAKI et al., 2005a).

2.6 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C, $^{1}H/^{13}C$ HSQC and $^{1}H/^{13}C$ HSQC-DEPT NMR spectra were acquired at 70 °C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient. The samples were acquired in D_2O with chemical shifts expressed as δ PPM, using the resonances of CH_3 groups of acetone (δ 30.2/ 2.22) as internal reference.

The degree of methyl esterification (DE) was determined by ^{1}H NMR spectroscopy according Grasdalen, Bakoy & Larsen (1988). Briefly, the fraction was deuterium-exchanged three times by freeze-drying with D_2O solutions, finally dissolved in D_2O and transferred into 5-mm NMR tube. The ^{1}H NMR spectra were acquired at 70 ^{o}C, with 256 scans, on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{1}H at 400.13 MHz. Chemical shifts were expressed as δ

PPM, using the resonances of acetone at δ 2.22 as internal reference. All pulse programs were supplied by Bruker.

3. Results and discussion

The fresh pulp of açaí fruits (228 g) was freeze-dried, yielding moisture of approximately 23%. The dried pulp powder (185 g) was then defatted with chloroform-methanol (1:1) in a Sohxlet apparatus, yielding approximately 20% of nonpolar compounds in açaí pulp. The defatted residue (148 g) was then submitted to successive extraction with water at 100ºC, and the extracted polysaccharides (fraction AW, 2.7% yield) recovered by EtOH precipitation and dialysis (Fig. 1).

As the first step of purification, fraction AW was submitted to freeze-thawing treatment, giving rise to a precipitated fraction (PAW, 0.6 % yield) and a supernatant fraction (SAW, 2.0% yield). Proteins from fraction SAW, were precipitated with 20% TCA, and the polysaccharide fraction SSAW was recovered in the supernatant portion (1.0% yield).

The monosaccharide analysis of SSAW indicated that it was composed mainly of uronic acids (44.6%), arabinose (16.5%), galactose (16.6%) and glucose (11.4%) (Table 1). Once SSAW had an heterogeneous elution profile in HPSEC analysis (data not shown), it was further fractionated through ultrafiltration membranes with 50 and 100 kDa cutoffs (Fig. 1). This procedure originated fractions 50E (eluted in 50 kDa cutoff membrane), 100E (eluted in 100 kDa cutoff membrane) and 100R (retained in 100 kDa cutoff membrane).

When analyzed through HPSEC, fraction 50E eluted as single peak (Fig. 2A), with a molecular mass of 22 kDa (*dn/dc* = 0.161). The main monosaccharides in 50E were uronic acids, wich comprised 77% of total sugars analysed, followed by glucose (13.6%) and mannose (9.2%) (Table 1). Indeed, the ^{13}C-NMR analysis (Fig. 3A) presented the main signals of 4-*O*-linked α-D-galacturonic acid. The signals at δ 100.1 and δ 99.3, corresponded to anomeric carbons of esterified and unesterified

units of α-D-Gal*p*A, respectively. Their respective C-6 signals were seen at δ 170.9 and δ 174.7, from methyl ester carbonyl carbons and carboxyl carbons. Signals of methyl carbons of esterified carbonyls in Gal*p*A units appeared at δ 52.8. The remaining carbons of the α-D-Gal*p*A ring were seen at δ 78.6 (*O*-substituted C-4), δ 70.5 and δ 71.6 (C-5 from esterified and non-esterified units, respectively), δ 68.8 (C-3) and δ 68.3 (C-2). Due to the presence of methylesterified α-D-Gal*p*A units, the degree of methyl esterification was determined by ^{1}H NMR spectroscopy. A value of 88% was found characterizing this polymer as a high-methoxyl (HM) pectin. The assignments are in agreement with published literature data (CIPRIANI et al., 2009b; XU et al., 2010). Moreover, due to the presence of a signal at δ 102.4 (Fig. 3A) and mannose and glucose in the monosaccharide composition (Table 1), an ^{1}H/^{13}C HSQC analysis was performed to investigate if another polymer, other than the homogalacturonan could be present in fraction 50E (Fig. 3B). The signal at δ 102.4/4.53 was assigned to C-1/H-1 of 4-*O*-linked β-D-Glc*p* units, and that at δ 100.1/4.75 to C-1/H-1 of 4-*O*-linked β-D-Man*p* units (GUO et al., 2012) indicating the presence of small amounts of a mannoglucan in fraction PF. Once fraction 50E presented a narrow peak in HPSEC analysis, this mannoglucan should have the same molecular weight of the above described HM homogalacturonan. It's noteworthy that mannoglucans have been previously reported for other Arecaceae family members, such as *Arecastrum romanzoffianum* (SILVA; PARENTE, 2010a) and *Syagrus oleraceae* (SILVA; PARENTE, 2010b).

Fractions originated from ultrafiltration through 100 kDa cutoff membrane, namely 100E and 100R, presented as main monosaccharides uronic acids, arabinose and galactose in 1.8:1.0:1.2 and 1.0:1.5:2.1 ratios, respectively (Table 1). When analyzed through HPSEC, only fraction 100E presented a homogeneous elution profile (Fig. 2A). However, both fractions presented similar ^{13}C-NMR spectra (Fig. 4A, B), indicating the presence of similar pectic polysaccharides, however with different molecular weights. In the spectra, besides the signals of a partially methylesterified (1→4)-linked-α-D-galacturonic acid (substituted and unsubstituted anomeric carbons at δ 99.9 and δ 99.3, respectively), anomeric signals of α-L-Ara*f* (δ 109.1 and δ 107.6) and *β*-D-Gal*p* (δ 103.3) were also observed. These data suggest the presence of an arabinogalactan (AG) in fraction 100E, as previously identified by Holderness et al. (2011) in açaí aqueous extract.

To further purify the AG from the homogalacturonan, fraction 100E was subjected to treatment with Fehling solution, giving rise to fractions SF (Fehling supernatant) and PF (Fehling precipitate). This strategy was highly efficient to precipitate the homogalacturonan, once the ^{13}C-NMRof PF fraction (data not shown) presented only the six signals of 4-*O*-linked α-D-Gal*p*A (δ 99.3, δ 68.3, δ 68.8, δ 78.6, δ 70.5 and δ 174.7, from C1-C6, respectively), while anomeric signals of the homogalacturonan previously observed at δ 99.9 and δ 99.3 in fraction 100E (Fig. 4A) were not present in fraction SF (Fig. 5). Both fractions (PF and SF) remained homogeneous when analyzed through HPSEC (Fig. 2B) and presented a similar molecular mass of 45 kDa (*dn/dc* = 0.147).

Monosaccharide composition of SF shows that it was composed mainly of arabinose (31.3%) and galactose (47.2%), indicating that the AG remained soluble in Fehling solution, as expected. These results were corroborated with methylation data (Table 2). Methylated derivatives from the galactan main chain, 2,3,4-Me_3-Gal-ol-acetate, 2,4,6-Me_3-Gal-ol-acetate and 2,4- Me_2-Gal-ol-acetate, from 6-*O*-, 3-*O*- and 3,6-*O*-linked Gal*p* units, respectively, were found in a 3.4:1.0:3.1 ratio. Thus, the AG present in fraction SF can be classified as a type II arabinogalactan (AGII). While a lower content of 3-*O*- than 3,6-di-*O*-linked Gal*p* units is an usual finding for type II arabinogalactans (CAPEK, 2008; CIPRIANI et al., 2006; DONG; FANG, 2001; DUAN et al., 2003; GOELLNER et al., 2011; ODONMAŽIG et al., 1994; PONDER; RICHARDS, 1997; SIMAS-TOSIN et al., 2012; WANG et al., 2005a; WILLFÖR et al., 2002). It is worth noting that a higher percentage of 6-*O*- than 3-*O*-linked Gal*p* units in AGII, as observed herein for açaí AGII, has been observed in few plant sources until now, where in some cases a (1→6)-β-galactopyranose backbone has been proposed (CAPEK, 2008; DONG; FANG, 2001; KIYOHARA; YAMADA; OTSUKA, 1987; OLIVEIRA et al., 2013; RAJU; DAVIDSON, 1994; WAGNER; JORDAN, 1988). Concerning fruits, up to now, pectic AGII has only been recently isolated and chemically characterized from pulp of peach (SIMAS-TOSIN et al., 2012) and starfruit (LEIVAS; IACOMINI; CORDEIRO, 2016). In the latter, as mentioned above, its AG-II also presented high amounts of (1→6)-linked Gal*p* units

In the methylation analysis, the arabinose residues that composed the AGII side chains were also observed mainly 5-*O*- (13.7%) and 3,5-*O*-linked (9.7%), due to

the presence of the derivatives 2,3-Me_2-Ara-ol-acetate and 2–Me-Ara-ol-acetate, respectively.

Type II arabinogalactans can be found associated or not to pectic polymers through type I rhamnogalacturonans (ASPINALL, 1973). Derivatives from the type I rhamnogalacturonan where the AGII is attached were also observed in fraction SF. The methylated derivatives 3,4-Me_2-Rha-ol-acetate, 3-Me-Rha-ol-acetate and 2,3,6-Me_3-Gal-ol-acetate arose from 2-*O*-linked Rha*p* (6.1%), 2,4-*O*-linked Rha*p* (2.8%) and the carboxyreduced 4-*O*-linked Gal*p*A (11.4%), respectively.

The $^{1}H/^{13}C$ HSQC-DEPT spectrum of SF is presented on Figure 5 and confirmed the presence of an AGII. Resonances from 3,6-*O*-linked β-D-Gal*p* and 6-*O*-linked β-D-Gal*p* units can be observed at δ 103.0/4.49 (C1/H1) and δ 69.3/4.04 (C6/H6 – $^{1}H/^{13}C$ HSQC-DEPT NMR inverted). The anomeric signal at δ 103.6/4.69 was attributed to 3-*O*-linked β-D-Gal*p* units with C3/H3 at δ 80.1/3.70. The latter was overlapped with the C3/H3 signal from 3,6-*O*-linked β-D-Gal*p* units. Anomeric signals of terminal, 5- and 3,5-*O*-linked α-L-Ara*f* units can be observed at δ 109.1/5.25, δ 107.4 /5.09 and δ 107.1/5.15, respectively. Moreover, inverted C5/H5 signals in $^{1}H/^{13}C$ HSQC-DEPT NMR experiment appeared at δ 61.3/3.78 for terminal α-L-Ara*f* and δ 66.6/3.87-3.80 for 5-*O*- and 3,5-*O*-linked α-L-Ara*f* units. Finally, signals from the type I rhamnogalacturonan could be observed in SF. Anomeric signals at δ 98.3/5.30 and δ 97.6/5.06 were assigned to α-L-Rha*p* and α-D-Gal*p*A units, respectively. While the C6/H6 signal at δ 16.5/1.24 was attributed to the 2-*O*-linked α-L-Rha*p*, the signal at δ 16.5/1.31 was attributed to the 2,4-*O*-linked α-L-Rha*p* units from the rhamnogalacturonan where the AGII is probably attached. The assignments are in agreement with published literature data (CAPEK et al., 2010; LIU et al., 2014; NASCIMENTO et al., 2013a; SHAKHMATOV; ATUKMAEV; MAKAROVA, 2016).

Overall, the detailed chemical analysis of açaí water soluble polysaccharides indicated the presence of a HM homogalacturonan, a type II arabinogalactan and small amounts of a glucomannan. It has previously been reported by Holderness et al. (2011) that a polysaccharide fraction from açaí containing an AGII induced innate immune responses both *in vitro* and *in vivo*. Notably, studies with type II arabinogalactans from a variety of sources demonstrated that they play a major role as immunostimulants (POPOV; OVODOV, 2013). Although there is no clear

information available about the structural requirements of arabinogalactans in order to have an optimal effect on promoting activity of different immune cells, the presence of 3,6-*O*-linked Gal*p* units were suggested to be of special significance (INNGJERDINGEN et al., 2007). However, some reports indicated that the presence of 6-*O*-linked Gal*p* units may be responsible for the expression of immunological activity as observed in *Plantago major* L (SAMUELSEN et al., 1996) and *Diospyros kaki* (DUAN et al., 2003). Thus, high amounts of 6-*O*- and 3,6-*O*-linked linked Gal*p* units rather than 3-*O*-linked Gal*p* units as found in AGII fraction from açaí berries may have contributed to the previously reported immunomodulatory effects of their polysaccharides. These findings contribute to establish structural *vs.* biological relationships of polysaccharides, and expand our knowledge about pectic polymers present in açaí.

Additionally, this work brings new insights into the structure of polysaccharides present in the cell walls of Arecaceae members. Despite being commelinid monocots, members of this family have a distinct cell wall composition, similar to that of non-commelinid and dicotyledons. Commelinid monocots have mainly glucuronoarabinoxylans in their cell walls and only small amounts of pectic polysaccharides and xyloglucans (CARNACHAN; HARRIS, 2000; HARRIS, 2005). On the other side, non-commelinid monocots and dicotyledonous primary cell walls typically have pectic polysaccharides as the predominant non-cellulosic polysaccharides, together with smaller amounts of xyloglucans and heteroxylans (CARNACHAN; HARRIS, 2000; HARRIS, 2005).

Herein the pectic polysaccharides were extracted from açaí pulp with hot water and obtained in 1.0% yield (deproteinized fraction SSAW). Furthermore, it is worth noting that in our lab, while studying hemicellulosic polysaccharides from açaí extracted with aq. 10% KOH (obtained in 5.9% yield), a linear (1→4)-β-D-xylan was found as the majority (70%) of alkaline extract and 4.2% of the açaí pulp dry matter. The presence of an arabinoxylan has also been observed in the açaí alkaline extract (data not shown). Thus, in terms of yields, we found higher amounts of xylans than pectic polysaccharides in açaí cell walls. This is in disagreement with the previous finding that palms have pectic polysaccharides being the predominant non-cellulosic cell wall polysaccharides. However, the açaí pulp was isolated from ripe fruits and

the proportion of pectic polysaccharides may have decreased during ripening. This hypothesis should be further confirmed. In a previous work, we studied the polysaccharides from the pulp of buriti (*M. flexuosa*) fruits, also from Arecaceae family (CANTU-JUNGLES et al., 2015; CORDEIRO et al., 2015) and encountered pectic e hemicellulosic polysaccharides in equal amounts. Thus, further studies on polysaccharides of Arecaceae members should be encouraged to unveil their primary cell wall chemistry.

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Table 1 - Monosaccharide composition of fractions obtained from aqueous extraction of açaí berries (*E. oleracea*).

Fraction	Neutral sugars[a]					Uronic acid[b]
	Rha	Ara	Man	Gal	Glc	
SSAW	4.4	16.5	6.5	16.6	11.4	44.6
50E	tr [c]	Tr	9.2	tr	13.6	77.0
100E	4.3	23.3	tr	28.8	tr	41.9
100R	8.6	30.3	tr	41.2	tr	19.5
SF	8.9	31.3	tr	47.2	tr	12.0

[a] % of peak area relative to total peak area, determined by GC-MS.

[b] Determined using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

[c] Trace amounts (<1%).

Table 2 - Linkage types based on analysis of partially *O*-methylalditol acetates obtained from methylated type II arabinogalactan (fraction SF) from açaí berries (*E. oleraceae*).

Partially *O*-methylalditol acetate	**SF[b]**	**Linkage type[c]**
2,3,5-Me_3-Ara[a]	8.2	Ara*f*-(1→
2,3-Me_2-Ara	13.7	→5)-Ara*f*-(1→
2–Me-Ara	9.7	→3,5)-Ara*f*-(1→
2,3,4,6-Me_4-Gal	18.9	Gal*p*-(1→
2,3,4-Me_3-Gal	13.2	→6)-Gal*p*-(1→
2,4,6-Me_3-Gal	3.9	→3)-Gal*p*-(1→
2,4- Me_2-Gal	12.1	→3,6)-Gal*p*-(1→
3,4- Me_2-Rha	6.1	→2)-Rha*p*-(1→
3-Me-Rha	2.8	→2,4)-Rha*p*-(1→
2,3,6-Me_3-Gal[d]	11.4	→4)-Gal*p*-(1→

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-Methylarabinitolacetate, etc.

[b] % of peak area of *O*-methylalditol acetates relative to total area, determined by GC-MS.

[c] Based on derived *O*-methylalditol acetates.

[d] From carboxyreduced Gal*p*A

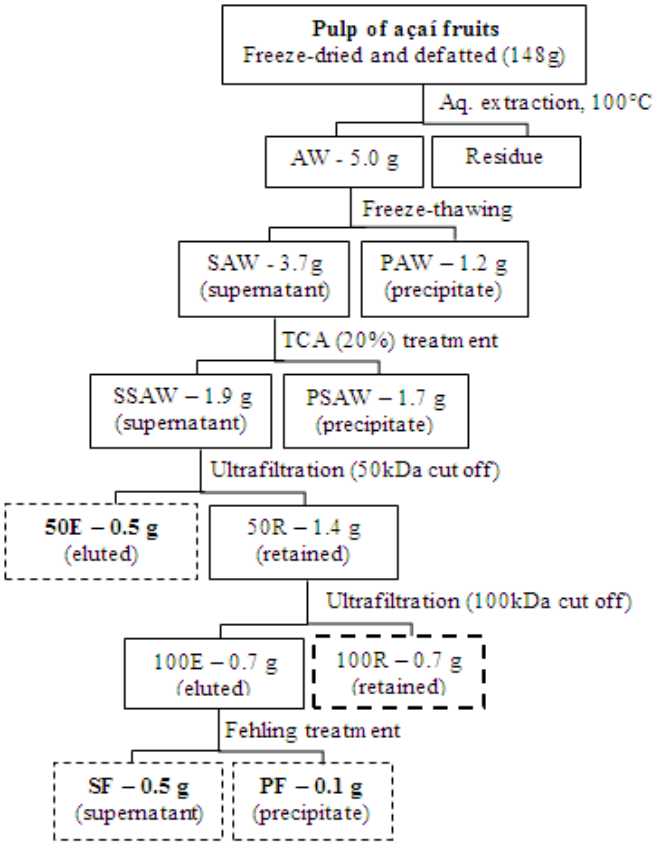

Figure 1. Scheme of extraction and fractionation of water extracted polysaccharides from açaí pulp *(E. oleracea)*.

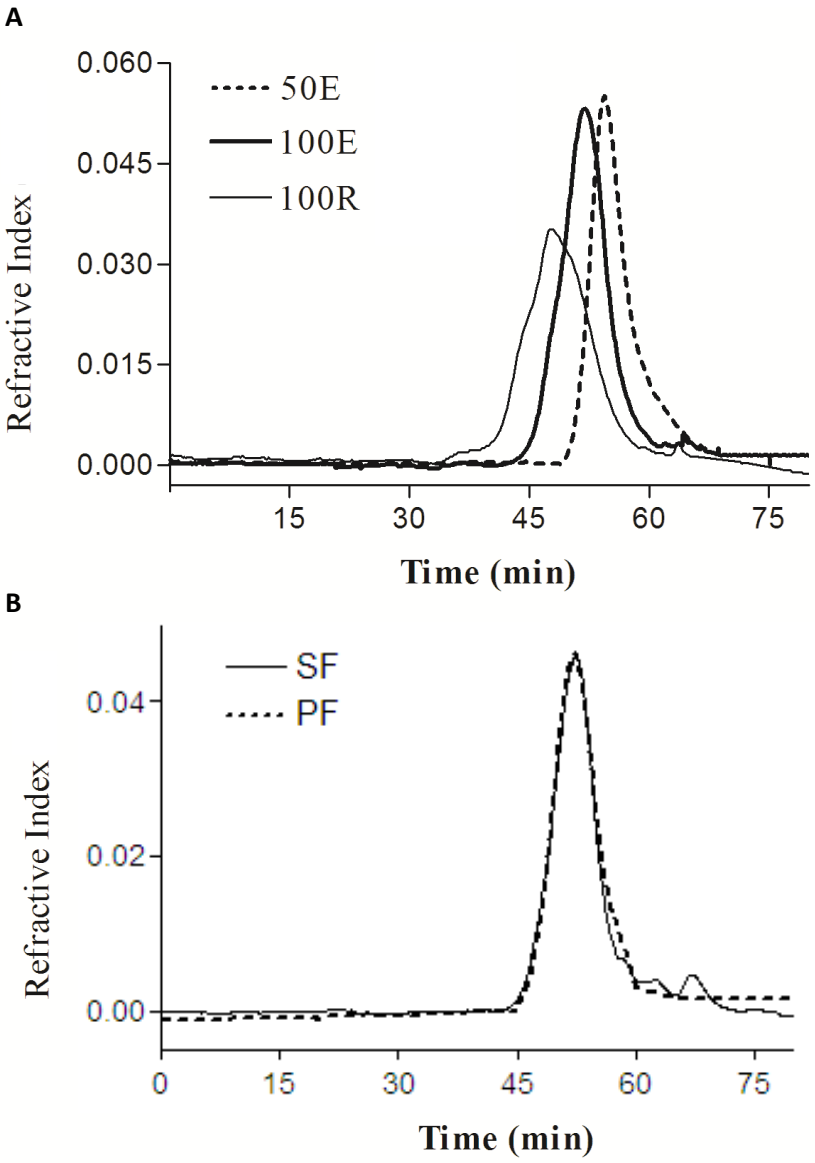

Figure 2. HPSEC elution profile of fractions 50E, 100E and 100R (A) and fractions PF and SF (B) obtained from açaí pulp (refractive index detector).

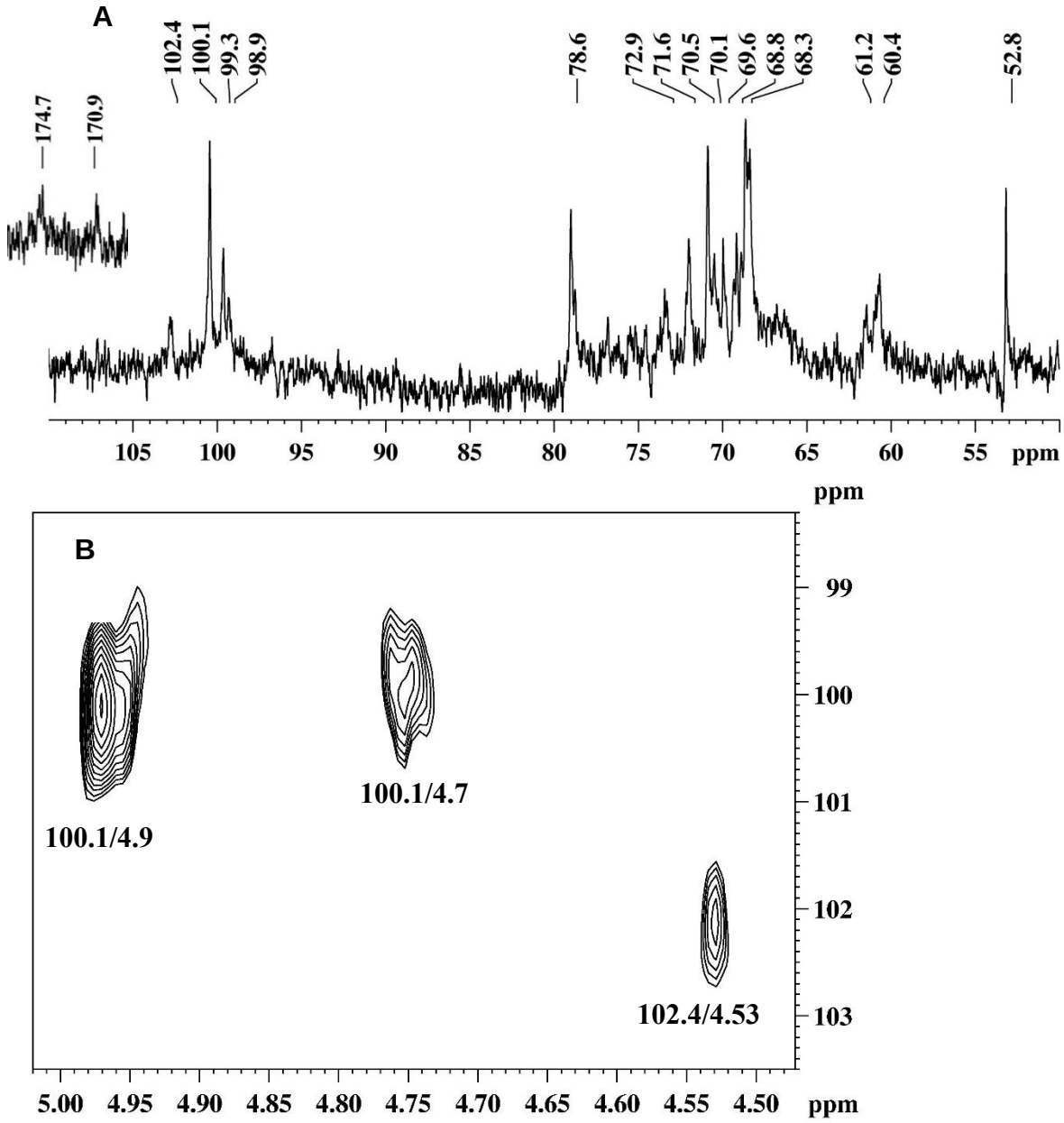

Figure 3. ^{13}C-NMR spectrum (A) and anomeric region from ^{1}H/^{13}C HSQC spectrum (B) of fraction 50E in D_2O at 70 ºC.

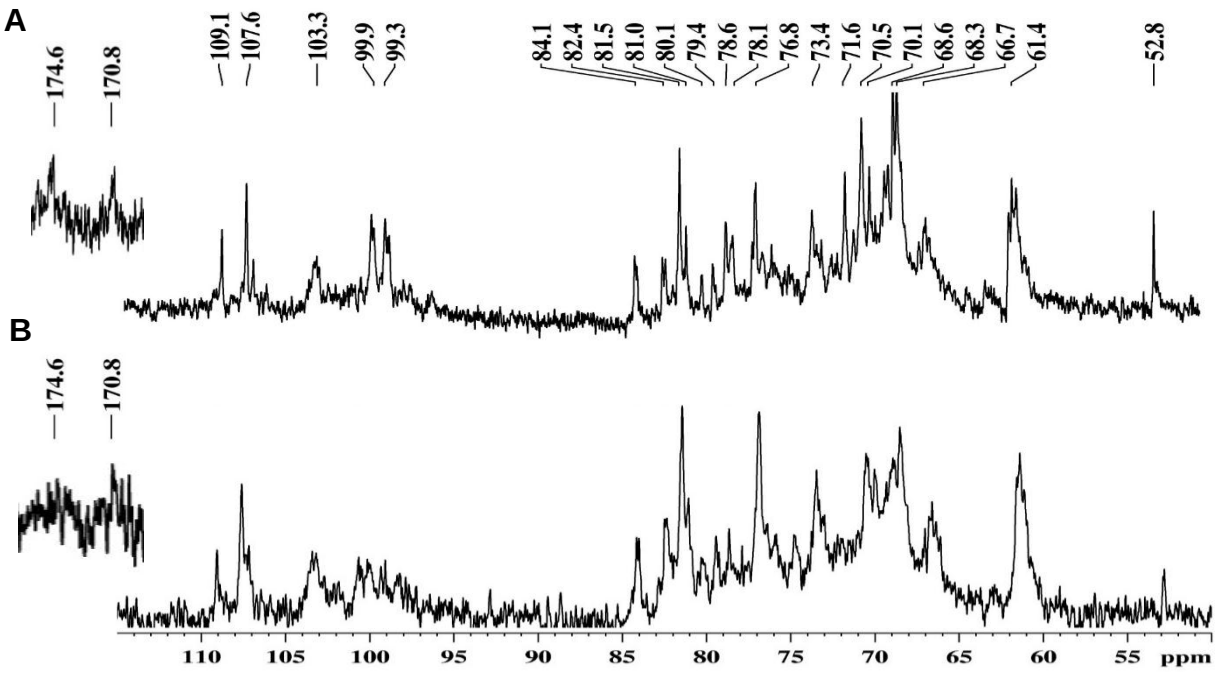

Figure 4. ^{13}C-NMR spectra of fractions 100E (A) and 100R (B) in D_2O at 70 ºC.

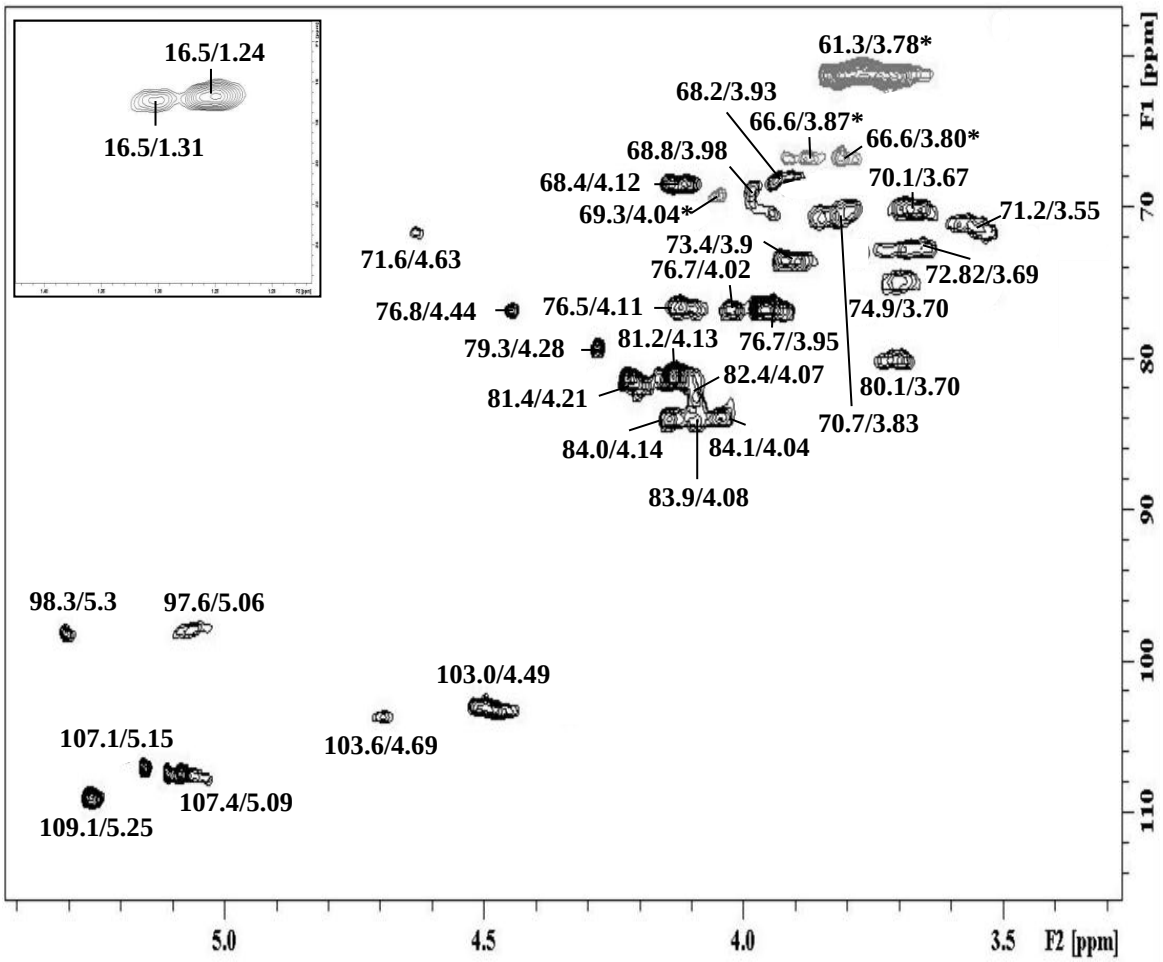

Figure 5. $^{1}H/^{13}C$ HSQC-DEPT spectrum of fraction SF in D_2O at 70 ºC. Inverted signals in DEPT experiment are marked with an asterisk (*).

ARTIGO IV

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Isolation and characterization of a xylan with industrial and biomedical applications from edible açaí berries (*Euterpe oleraceae*)

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ABSTRACT

Xylan chemical features largely determine physical and biological properties that dictate its uses in the industry. In this work, we describe the occurrence, purification and partial characterization of a xylan in edible açaí berries (*Euterpe oleraceae*), using a fairly simple and inexpensive method of purification from alkaline açaí extract. A mainly linear (1→4)-β-D-xylan was found as the majority (70%) of alkali extract and 4.2% of the açaí pulp dry matter. This represents the biggest source of xylan found so far in a fruit pulp and could be suitable for applications in the industry and biomedical field.

Keywords: Açaí berries; *Euterpe oleraceae;* xylan.

1. Introduction

Xylan polysaccharides have gained increasing attention in the last decades due to their functional properties and applications in the food and non-food areas. Structurally, xylans are a diverse group of polysaccharides with the common feature of a backbone of β-(1→4)-linked xylopyranosyl units (VIIKARI et al., 2002). Depending on the source and extraction methodology, different side chains may be attached to the xylan backbone such as α-D-glucuronic acid, 4-*O*-methyl-α-D-glucuronic acid and some neutral sugar units such α-L-arabinose, α-D-xylose and α-D-galactose. Side groups like acetyl groups, phenolic acids such as ferulic and coumaric acids can also be found (HROMÁDKOVÁ; KOŠT'ÁLOVÁ; EBRINGEROVÁ, 2008; TELEMAN et al., 2002; VIIKARI et al., 2002).

The molecular size, degree of branching and monosaccharides that compose side chains largely determine physical and biological properties that dictate xylan uses in the industry. Debranched or linear polymers are used for application as surface modifiers for cellulosic materials (BOSMANS et al., 2014) or to form hydrogels as entrapment matrices for slow delivery of bioactive substances (CHIMPHANGO; VAN ZYL; GÖRGENS, 2012). On the other hand, arabinose branched xylans, due to the high water binding capacity and the formation of viscous solutions in water, influence biotechnological processes in bread baking (ROSICKA-KACZMAREK et al., 2015). Moreover, pentosan polysulfate, an FDA-approved oral medicine, known for its anticoagulant, anti-inflammatory and anticancer effects, and for lowering cholesterol and triglyceride levels is prepared by addition of sulfate groups in beechwood xylan (DOCTOR; SAULS, 1983; SCHUCHMAN et al., 2013).

Xylans may be also hydrolyzed through enzymes and thermal and/or acid conditions to give rise to xylooligosaccharides (XOS). These can be used as prebiotics, selectively stimulating the growth of beneficial gut microbiota (SAMANTA et al., 2015). Other health benefits already investigated for XOS include reduction in blood glucose and cholesterol, reduction of pro-carcinogenic enzymes in the gastrointestinal tract, enhanced mineral absorption from large intestine and immune-stimulation (SAMANTA et al., 2015; VAZQUEZ et al., 2000). Indeed, in countries like

Japan, XOS have already been incorporated into some foods, predominantly in prebiotic drinks (CRITTENDEN; PLAYNE, 1996; VÁZQUEZ et al., 2000).

Good sources of xylan include agricultural crops such as straw, sorghum, sugar cane, corn stalks and cobs, hulls and husks from starch production, as well as forest and pulping waste products from hardwoods (EBRINGEROVÁ; HROMÁDKOVÁ, 1999). However, the occurrence of such polymers in high yields in fruits is rarely reported on the literature. Moreover, in many xylan sources, the presence of variable amounts of other polymers such as cellulose, starch, pectin, arabinogalactan, xyloglucan and galactomanan as well as proteins and phenolics may result in the need of expensive and/or multistep procedures to obtain a xylan with a desirable purity degree (EBRINGEROVA; HEINZE, 2000).

Thus, in this work, we describe the occurrence, purification and partial characterization of a xylan in edible açaí berries (*Euterpe oleraceae*), using a fairly simple and inexpensive method of purification from açaí alkaline extract.

2. Materials and methods

2.1 Plant material

Ripe fruits of açaí (*E. oleraceae*) were purchased at local market in Belém, State of Pará, Brazil.

2.2 Extraction and purification of polysaccharides

The seeds were manually removed and the pulp (228.6 g) was freeze-dried and milled. Dried pulp powder was defatted with chloroform–methanol (1:1), in order

to remove lipids, pigments and other hydrophobic materials. The general polysaccharide extraction procedures were conducted according to Nascimento et al. (2013). Briefly, the polysaccharides were extracted from the residue with water at 100 ºC for 2 h (x 7, 1 L each). The aqueous extracts were clarified by centrifugation (3860*g*, 20 min at 25 ºC). The residue was then extracted three times (1 L each) with aq. 10% KOH, at 100 ºC for 2 h and the alkaline extracts were neutralized with acetic acid, dialyzed for 48 h with tap water, concentrated under reduced pressure and freeze-dried, to give fraction AK.

A freeze–thaw treatment was applied in fractions AK to separate cold-water soluble and insoluble fractions (Fig. 1). In this procedure, the sample was frozen and then thawed at room temperature. Insoluble polysaccharides (fraction PAK) were recovered by centrifugation (3860*g*, 20 min at 4 °C).

The yields were expressed as % based on the weight of dried açaí pulp (185 g).

2.3 Sugar composition

Neutral monosaccharide components of the polysaccharides and their ratio were determined following the conditions employed by Cantu-Jungles et al. (2015).

Uronic acid contents were determined spectrofotometrically using the *m*-hydroxybiphenyl method (FILISETTI-COZZI; CARPITA, 1991).

2.4 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C-NMR spectrum of fraction PAK was acquired at 70 °C on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{13}C at 100.61 MHz, equipped with a 5-mm multinuclear inverse detection probe with z-gradient.

The samples were acquired in DMSO with chemical shifts expressed as δ PPM, using the resonances of DMSO-d_6 at δ 39.7 as internal reference.

3. Results and discussion

The pulp of açaí fruits was freeze–dried (yielding a moisture content of approximately 23%). The dried pulp powder (185 g) was then defatted with chloroform–methanol (1:1) in a Sohxlet apparatus, yielding nonpolar compounds at a content of approximately 20%. The defatted residue (148 g) was then submitted to successive extraction with hot water and 10% aq. KOH. The alkali-extracted polysaccharides (fraction AK) were recovered by dialysis (Fig. 1) with a 5.9% yield. It was submitted to a freeze–thaw treatment, that after centrifugation gave rise to a cold water soluble and to the cold water insoluble fraction PAK (4.2% yield).

Monosaccharide composition of fraction PAK presented mainly xylose (97.2%) and small amounts of uronic acids (2.8%), indicating the presence of a xylan in this fraction. The ^{13}C-NMR spectrum of fraction PAK is shown in Fig. 2. It presented five intense signals, a low-field C-1 signal at δ 101.7, indicating β-Xyl*p* units, and others at δ 75.6 (*O*-substituted C-4), 74.0 (C-3), 72.7 (C-2) and 63.2 (C-5), characteristic of linear (1→4)-β-D-linked xylan (CORDEIRO et al., 2015; KOVAČ et al., 1980).

It's noteworthy however, that branches of glucuronic acid occurred in very low proportion (only 2.8% according to uronic acids measurement), thus, the (1→4)-β-D-linked xylan found in fraction PAK is mainly linear. This very low substitution is unusual. In hardwoods 4-*O*-methyl glucuronic acid (MeGlcA) is found at a ratio of approximately 1:10 to xylose (MAGATON et al., 2011; SPIRIDON; POPA, 2008; TELEMAN et al., 2002). In grasses, the ratio of MeGlcA to xylose is around 2:10 (EVTUGUIN et al., 2003; IZYDORCZYK; DEXTER, 2008; WESTBYE et al., 2007). Low/no branched xylans are useful as surface modifiers for cellulosic materials to form hydrogels, as entrapment matrices for slow delivery of bioactive substances and may facilitate the production of XOS, once side chain specific enzymes are not required. Linear xylans are not commonly found in the nature and have been only

isolated from esparto grass (CHANDA et al., 1950), stalks of *Nicotiana tabacum* (EDA; OHNISHI; KATŌ, 1976), husks of guar seed (SAJJAN; SALIMATH, 1986), seed endosperms of *Opuntia ficus-indica* prickly pear fruits (HABIBI; MAHROUZ; VIGNON, 2002), pericarps of *Argania spinosa* fruit (HABIBI; VIGNON, 2005) and buriti (*Mauritia flexuosa*) pulp (CORDEIRO et al., 2015).

Common xylan sources such as corn bran and beechwood have yields of 3.0 to 54.0% and 1.8 to 23.9%, respectively, depending on the extraction technique (EBRINGEROVA; HEINZE, 2000). However, the variety of side chains may limit its applications or require further hydrolysis steps for branches elimination before industrial use. On the other hand, açaí xylans found in 4.2% yield in this work have a more linear structure and, to our knowledge, represent the biggest source of xylan found so far in a fruit pulp. Notably, the edible açaí berries represent an important crop in north regions of Brazil, with production reaching around 121,000 tons per year (ZERRER, 2012). Thus, the very low branched xylan from açaí berries could be suitable for applications in the industry and biomedical field. Furthermore, extraction methodologies to increase xylan yield were not the focus in this study and future research could be conducted to further optimize xylan extraction.

4. Conclusions

A mainly linear (1→4)-β-D-xylan was obtained through a rather simple process of freeze-thawing from açaí pulp alkaline extract. The (1→4)-β-D-xylan corresponded to the majority (70%) of alkaline extract and 4.2% of the açaí pulp dry matter.

Acknowledgements

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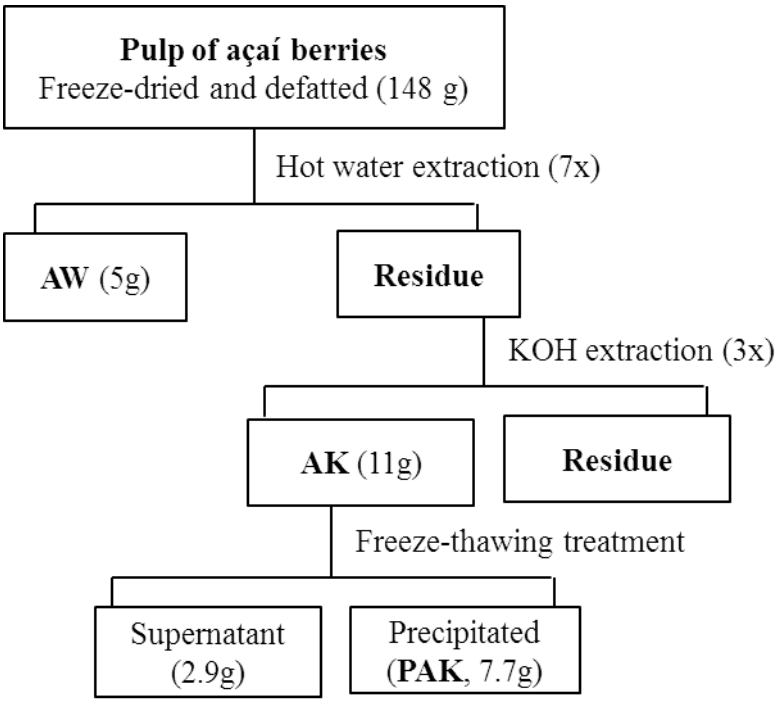

Figure 1. Scheme of extraction and fractionation of polysaccharides from the pulp of açaí fruits (*E. oleraceae*).

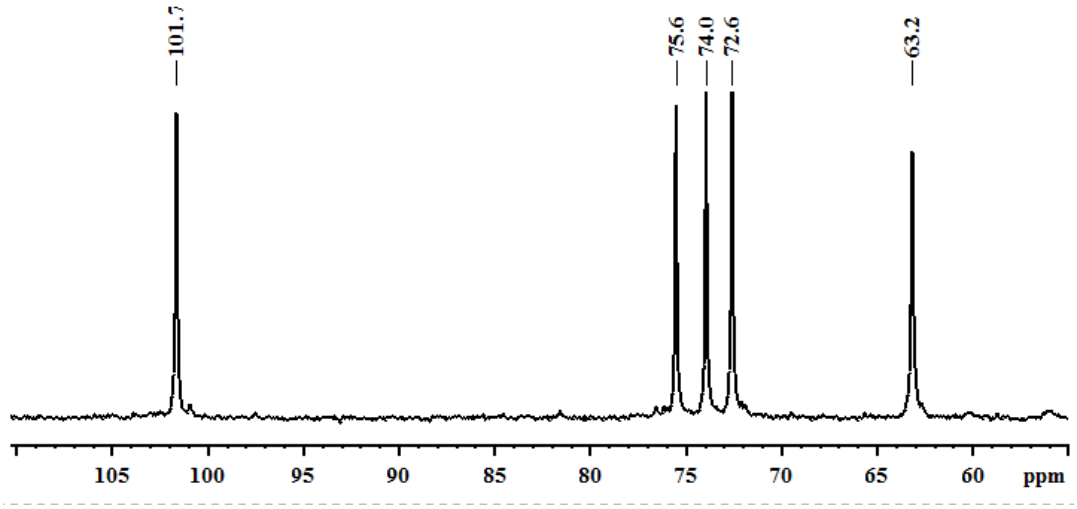

Figure 2. ^{13}C-NMR spectrum of fraction PAK, in DMSO at 70ºC, (chemical shifts are expressed as δ PPM) obtained from açaí pulp.

ARTIGO V

(Em fase de edição para submissão)

Microwave treatment changes the tridimensional structure of insoluble fibers and improves their fermentation profile by human gut microbiota

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ABSTRACT

Most of insoluble dietary fibers are known to be poorly fermented by the human gut microbiota. Since microwave (MW) treatment is capable to enhance the susceptibility of insoluble materials to enzymatic hydrolysis we tested if MW would enhance insoluble dietary fibers (xylan, arabinan and arabinoxylan) fermentability in an *in vitro* gut fermentation model. An increase in fermentation metabolites produced including the main short chain fatty acids (acetate, butyrate and propionate) could be observed for all tested dietary fibers after MW. Moreover, MW treatment increased xylan solubility and decrease arabinoxylan solubility. Finally, re-arrangments in tridimensional structure of dietary fibers were observed after MW.

Keywords: Insoluble dietary fibers; *in vitro* fecal fermentation; microwave treatment; dietary fiber tridimensional structure.

1. Introduction

Dietary fibers (DF) are carbohydrate polymers with ten or more monomeric units and lignin, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans (CODEX ALIMENTARIUS COMMISSION, 2009). When these polymers reach the large intestine, intestinal flora uses a range of carbohydrate hydrolyzing enzymes to partially or fully ferment them. Among the metabolites generated during this process significant amounts of short chain fatty acids (SCFAs) are produced, specially acetate, butyrate and propionate (SLAVIN, 2013). At both the colonic and systemic levels, fermentation and particularly SCFA production generate a variety of health benefits that include, but are not limited to the maintenance of gut integrity and barrier function, regulation of the intestinal transit and metabolism of fatty acids, glucose and cholesterol (BESTEN et al., 2013; KACZMARCZYK et al., 2012; SLAVIN, 2013).

The type and origin of DF greatly influences the site and degree to which it can be degraded, mainly depending on the degree of lignification, solubility and chemical structure (KNUDSEN, 2001). Notably, one of DF classification differentiates dietary components based on their solubility, and/or their fermentability. The water-insoluble/less fermented fibers include cellulose, hemicellulose and lignin while the water- soluble/well fermented fibers include pectin, gums and mucilages (DHINGRA et al., 2012). Although both soluble and insoluble DF can be degraded by intestinal bacteria at some extent, soluble DF are prone to be more accessible to hydrolytic enzymes, and thus to present an easier, more rapid and complete fermentation in the colon (BACH; HANSEN, 1991; FAO, 1998).

Chemical and physical modification of soluble and insoluble polymers can be performed for a variety of purposes. In this sense, the treatment of insoluble materials with microwave (MW) irradiation has gained attention in the last decades. Microwaves are uniformly absorbed by the suspension and cause vibration of the molecules that create rapid and uniform sample heating through friction occurring with dipole rotations (MANDAL; MOHAN; HEMALATHA, 2007). The use of MW as a pre-treatment to enhance enzymatic digestibility of lignocellulosic biomass in

combination with alkali and acids is well reported in the literature (HU; WEN, 2008; INTANAKUL; KRAIRIKSH; KITCHAIYA, 2003; ZHU et al., 2006). Because MW radiation results in the rupture of cell wall matrices, the technique has also been used for the extraction of phenolic compounds from a variety of materials (GALLO et al., 2010; SPIGNO; FAVERI, 2009), as well as hemicelluloses from agricultural by-products (WANG et al., 2007; YOSHIDA et al., 2010). Since MW treatment is capable to enhance the susceptibility of insoluble materials to enzymatic hydrolysis we tested if MW would enhance insoluble dietary fibers fermentability in an *in vitro* gut fermentation model. Furthermore, possible physical modifications caused by microwaving procedure such as shifts in solubility and tridimensional structure re-arrangements were investigated.

2. Materials and Methods

2.1 Substrates

The following previously characterized insoluble DF were utilized for MW treatment and further *in vitro* fecal fermentation analysis: a linear xylan extracted from açai berries (*Euterpe oleracea*) (CANTU-JUNGLES et al, 2016) and a linear arabinan and an arabinoxylan (AX) from tucumã (*Astrocaryum aculeatum*) (unpublished data). These polymers were chosen due to its high insolubility degree.

2.2 Microwave treatment

The xylan, arabinan and arabinoxylan samples were suspended in deionized water (10% w/v) and poured into MARSXpress™ digestion vessel (10 ml in 55 ml sized vessels) containing a magnetic stirring bar, and a minimum of 8 Xpress vessels were run at once for optimum temperature control. Tightly sealed vessels were then

subjected to MW radiation, under constant stirring, in the MARSXpress™ microwave (CEM Corporation, Mathews, NC, USA) at 800 W, 120 °C for 30 min. MW treated samples were freeze-dried for further analyses.

2.3 *In vitro* fecal fermentation

Batch fecal fermentation was performed as the methodology of Lebet et al. (1998) with some minor modifications (ROSE; PATTERSON; HAMAKER, 2010). The total carbohydrate in the samples was determined by the phenol-sulphuric acid method using xylose and/or arabinose as standards (DUBOIS et al., 1956). Each substrate (50 mg equivalent carbohydrate) was weighed in 3 test tubes for each time point such that all the analysis occurred in triplicate. Hydration of samples was performed by adding 4 mL of carbonate-phosphate buffer pH 6.8 ± 0.1 to each tube. Fructooligosaccharides (FOS - No. F8052, Sigma-Aldrich Inc., St. Louis, Mo., USA) and tubes without any added carbohydrate were used as positive and negative controls, respectively.

Fecal samples were obtained from 3 healthy volunteers who were on their routine diet and had not taken antibiotics within the previous 6 months. Fecal samples were collected in plastic bags that were sealed after escaping the air, transported on ice, and immediately placed inside an anaerobic chamber (10% H_2, 5% CO_2, and 85% N_2; BactronEZ, SHEL LAB, Cornelius, OR) where all further procedures were performed within 2 hours after collection. Fecal samples were pooled together and the fecal slurry prepared by homogenization with carbonate-phosphate buffer pH 6.8 ± 0.1 in a ratio of 1:3 (w/v) and further strained through 4 layers of cheese cloth. The filtrate (1 mL) was then inoculated with the hydrated carbohydrate sample and the controls. Tubes were sealed and incubated at 37 ºC in a shaking water bath. Immediately after incubation and after 4, 8, 12, and 24 h of fermentation, assigned tubes were removed from the water bath, and total gas volume was measured. To terminate microbial activity, copper sulphate solution (400 µL of 2.75 mg/mL) was added and pH of samples recorded. Aliquots (400 µL) were

combined with 100 µL of a mixture containing 50 mM 4-methyl-valeric acid (No. 277827- 5G, Sigma-Aldrich Inc., St. Louis, Mo., USA), 5% meta-phosphoric acid, and copper sulphate (1.56 mg/mL); mixed with a vortex mixer and stored at - 40 ºC until used for SCFAs analysis. To quantify SCFA content, frozen fermentation residues were thawed and centrifuged at 13,000 rpm for 10 min. An aliquot (0.2 µL) was injected into a GC-FID 7890A (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a fused silica capillary column (NukolTM , Supelco No. 40369-03A, Bellefonte, Pa.,USA). The initial oven temperature was held at 50°C for 2 min, ramped to 70 °C at a rate of 10 °C/min, to 85 °C at a rate of 3 °C/min, to 110 °C at a rate of 5 °C/min, to 290 °C at a rate of 30 °C/min, and finally held at 290 °C for 8 min. Helium was used as a carrier gas at a constant flow rate of 1 mL/min through the column.

2.4 Evaluation of physical changes generated by microwave treatment

2.4.1 Determination of Soluble Carbohydrate Content

In order to to evaluate changes solubility caused by MW treatment, the amount of soluble carbohydrates in native and MW-treated fractions was quantified. Initially, fractions were dispersed in water (25 mg/ml) at room temperature under stirring for 1 hour, centrifuged (10,000 rpm, 15 min) and the supernatant freeze dried and weighted. Percentage of soluble polysaccharides were calculated based on the sample total carbohydrate content measured by the phenol-sulfuric method (DUBOIS et al., 1956) in the samples before and after centrifugation (supernatant fraction only). Analyses were performed in triplicates and the content of soluble carbohydrates expressed as % of the total carbohydrate content.

2.4.2 Scanning Electron Microscopy (SEM)

The surface morphology of the polymers was investigated by scanning electron microscopy (SEM, Tescan Vega3 LMU). Prior to imaging, samples were placed on double-sided adhesive tape pieces, which were mounted on specimen holders. Samples were then coated by gold sputtering to impart electronic conductivity to the sample for imaging in a sputter coater SCD030 (Balzers Union, FL 9496).

2.5 Statistical analysis

Statistical differences among data were determined using GraphPad Prism software package version 7.0 (GraphPad Software, Inc., California, U.S.A.). Differences in gas, pH, and each SCFA produced at each time point among substrates were analyzed using repeated measures analysis of variance and differences between least square means were calculated using Tukey's multiple comparisons test.

3. Results and discussion

3.1 Fermentation profile of insoluble DF by the human gut microbiota *in vitro* before and after irradiation with microwaves

Native xylan, arabinoxylan (AX) and arabinan, together with negative (blank) and positive (FOS) controls were submitted to *in vitro* fecal fermentation and the generated metabolites profile from saccarolytic fermentation (SCFA) and amino acids

fermentation (BCFA) together with gas and pH, analyzed in 0, 4, 8, 12 and 24 hours of fermentation (Tables 1 and 2; Fig 1 and 2).

After 8, 12 and 24 hour fermentation, gas production and pH variations were similar or lower than the blank for all insoluble native polymers tested (xylan, AX and arabinan) (Table 1). Moreover, they induced to a total SCFA and BCFA production similar to the blank for the xylan and even lower than the blank for the AX and arabinan in all time points (Table 2, Fig 1). Thus, it seems like the AX and arabinan were not only poorly fermented, but also prevented the fermentation of other dietary fibers and proteins present in the fecal inocula. Previous studies also indicate that some fermentable polymers such as pectins may prevent/delay the utilization of other DF in *in vivo* models (TIAN et al., 2016a; TIAN et al., 2016b). However, these results were more related to microbial preferences and competition for substrates and thus, fermentation and SCFA production still occurred (TIAN et al., 2016a). In our findings however, the reduced fermentation profile compared to the blank indicates that the presence of the insoluble DF in the slurry could chemically or physically difficult the utilization or access of bacteria to fermentable substrates.

Because MW treatment is used to enhance the susceptibility of insoluble materials to enzymatic hydrolysis, we also evaluated the fermentation profile of microwave treated xylan (xylan-MW), arabinoxylan (AX-MW) and arabinan (arabinan-MW). Fermentation of xylan-MW and arabinan-MW significantly reduced pH and increased the gas production if compared to its native forms in all time points (Table 1). Likewise, the total SCFA production in 24 hours was increased 3.5 times for the xylan-MW, 5 times for the AX-MW and 4.1 times for the arabinan-MW in comparison to its native forms (xylan, AX and arabinan, respectively) (Table 2, Fig. 1A). The absolute total SCFA increase was higher for xylan-MW (28.7mM/ 50mg carbohydrate), followed by AX-MW (23.1mM/ 50mg carbohydrate) and arabinan-MW (12.9mM/ 50mg carbohydrate) (Table 2). BCFA production was also increased in microwaved arabinoxylan (AX-MW) and arabinan (arabinan-MW) reaching concentrations similar to FOS in 24 hours (Fig 1B). For xylan-MW however, a 66.6% decrease in BCFA production can be observed if compared to the original sample (xylan), reaching even lower concentrations than the negative control (blank) (Fig 1B, Table 2). Overall, BCFA represented from 0.3% to 5.2% of the total SCFA produced

in all samples, in comparison to 5.5% in the blank. These data suggest that even in the samples with increased BCFA production after MW, this was a result of greater overall fermentative activity and not a tendency towards putrefactive fermentation.

Regarding specific short chain fatty acids, acetate, butyrate and propionate were produced in higher amounts than valerate and caproate (Fig 2). Increases in acetate and propionate production between native and MW treated samples were of 3.1 and 2.7 times for the xylan, 4.7 and 5.1 times more for the arabinoxylan and 4.3 and 3.3 times more for arabinan, respectively (Fig 2A and B, Table 2).Absolute increase values were higher for the xylan, followed by AX and lastly the arabinan, indicating that the rate to which MW treatment affected SCFA production depended on the fermentability of native polymers and their chemical structure (Table 2). Indeed, although all MW treated samples presented an increased butyrate production, a surprisingly large intensification was observed for xylan after microwaving treatment (7 times more than the native polymer) being only 10.2% lower than that of FOS after 24 hours fermentation (Fig. 2C, Table 2). When analyzing proportions among the 3 main SCFA in microwaved samples in comparison to the blank and FOS it can be observed that the fermentation of xylan-MW generated the highest proportions of butyrate among all samples (Fig 3). The preferable production of butyrate has promising effects for treatment of colon disorders including irritable bowel disease and colon cancers (CANANI et al., 2011).

Moreover, while FOS lead to butyrate production mostly in the initial 4 hour fermentation, the xylan-MW presented a more continuous butyrate production with around half of total butyrate being produced after 8 hours fermentation (Fig 2C). Extrapolating for *in vivo* systems, the slow production of butyrate presented by the Xylan-MW allows this fatty acid to be delivered in both, proximal and more distal parts of the colon. The availability of SCFA throughout the entire length of colon is desirable because 50% to 60% of large intestinal cancer occurs in the distal colon (CUMMINGS, 1997).

MW treatment of all samples also induced to a higher production of valeric acid (Fig 2D, Table 2) but only microwaved xylan (xylan-MW) had a higher caproic acid production if compared to its native form (xylan) (Fig 2E, Table 2). It’s noteworthy,

that valeric and caproic acids together comprise less than 12% of the total SCFAs produced in all tested samples.

3.2 Physical changes generated by microwave treatment

It's well known that soluble polysaccharides are better fermented than insoluble polymers. Once MW treatment was capable to increase SCFA production during *in vitro* fermentation for all samples, we evaluated whether this phenomena was caused by an increase on the polymers solubility. The carbohydrate solubility of samples was differently affected by MW depending on the type of DF (Figure 4). A significant increase (from 0.05% to 9.3%) in the xylan solubility could be observed, while for the arabinoxylan, carbohydrates solubility decreased from 16.9% to 11.8%. It's possible that MW treatment induced samples autohydrolysis at some extent affecting its solubility degree and fermentation profile. During MW procedure hydronium ions and *in situ* generated organic acids at high processing temperatures (160–220 ^{o}C) may partially hydrolyze hemicellulosic polymers and yield soluble hydrolysates (MOURE et al., 2006). In this sense, while xylan could've been hydrolyzed to smaller molecule size increasing its solubility, MW treatment in arabinoxylan, could have caused hydrolysis of arabinose side chains, reducing even more its solubility degree. Moreover, MW treatment did not produce significant changes in arabinan solubility, while still increased its fermentation profile. Thus, solubility degree alone does not fully explain the observed shifts in short chain fatty acids production.

To better understand physical factors influencing the fermentability in tested polymers, topological features of the polysaccharides before and after microwave treatment were studied using electron microscopy. Comparing between native and microwaved samples, a discrete increase of particles size can be observed for the arabinan and a decrease for the arabinoxylan, with the similar rocky-like agglomerate feature in both polymers before and after microwaving (Fig. 5). Particle size calculations were limited due to the heterogeneous aspect of these samples. For the

xylan, however, while the native form presented a flat surface, microwaving treatment cause the molecules tridimensional structure to re-arrange in a coin-like manner that was pretty consistent through the sample. The calculated diameter for the coin-like particles was of ~1.1µm. The homogeneous coin-like microstructure observed could've been generated through the controlled and constant particles agitation during MW. That may partially explain the increased fermentation profile of xylan MW-treated sample, once it may have increased the access of bacteria to more portions of the polymer.

4. Conclusion

In sum, native xylan, AX and arabinan, were not fermented on the *in vitro* fecal fermentation model. After MW treatment of these samples, an increase in the main SCFA production (acetate, butyrate and propionate) could be observed. An unexpected large amount of butyrate was slowly produced through fermentation for xylan after microwaving (xylan-MW). For the latter, changes in solubility degree and polymer tridimensional microstructure occurred and may be related to the increased fermentation profile of MW treated sample during *in vitro* fecal fermentation.

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Table 1 – Gas production (ml/50 mg carbohydrate) and pH during *in vitro* fecal fermentation*

Time (h)	Blank	FOS	Xylan	Xylan (MW)	AX	AX (MW)	Arabinan	Arabinan (MW)
Gas								
4	2.5 (0.3)^{AB}	5.6 (0.2)^{C}	3.1 (0.1)^{DE}	4.0 (0.0)^{F}	2.7 (0.2)^{AD}	3.9 (0.1)^{F}	2.7 (0.2)^{BD}	2.8 (0.3)^{BE}
8	3.1 (0.1)^{A}	7.8 (0.1)^{B}	3.1 (0.8)^{A}	5.3 (0.1)^{C}	3.1 (0.1)^{A}	3.9 (0.1)^{D}	3.0 (0.1)^{A}	3.0 (0.0)^{A}
12	2.8 (0.8)^{A}	10.0 (0.0)^{B}	3.2 (0.3)^{A}	6.9 (0.1)^{C}	3.4 (0.1)^{A}	4.9 (0.1)^{D}	3.2 (0.0)^{A}	3.3 (0.2)^{A}
24	3.2 (0.3)^{A}	10.2 (0.3)^{B}	2.4 (0.2)^{C}	7.2 (0.0)^{D}	3.3 (0.1)^{A}	4.9 (0.1)^{E}	3.1 (0.1)^{A}	3.0 (0.0)^{A}
pH								
4	7.34 (0.05)^{A}	6.79 (0.02)^{B}	7.33 (0.02)^{A}	7.02 (0.02)^{C}	7.36 (0.01)^{A}	7.15 (0.08)^{D}	7.42 (0.03)^{A}	7.37 (0.02)^{A}
8	7.33 (0.02)^{A}	6.75 (0.01)^{B}	7.37 (0.04)^{AC}	6.93 (0.01)^{D}	7.36 (0.01)^{AC}	7.16 (0.04)^{E}	7.40 (0.02)^{C}	7.37 (0.01)^{AC}
12	7.39 (0.04)^{AB}	6.71 (0.01)^{C}	7.42 (0.02)^{A}	6.98 (0.01)^{D}	7.35 (0.01)^{B}	7.17 (0.01)^{E}	7.39 (0.01)^{AB}	7.37 (0.01)^{B}
24	7.42 (0.02)^{A}	6.76 (0.01)^{B}	7.39 (0.05)^{A}	6.91 (0.02)^{C}	7.39 (0.02)^{A}	7.21 (0.02)^{D}	7.43 (0.01)^{A}	7.40 (0.01)^{A}

*Means (standard deviation) at 0 h for all the samples: gas= 1.6 mL (0.1) ; pH= 7.11 (0.01); BCFA= 0.1 (0.0). Means (SD) within row with the different capital letter superscripts are significantly different ($P<0.05$).

Table 2. Short chain fatty acid and branched chain fatty acid production (µmol/50 mg carbohydrate) during *in vitro* fecal fermentation*

Time (h)	Blank	FOS	Xylan	Xylan (MW)	AX	AX (MW)	Arabinan	Arabinan (MW)
Total SCFA								
4	9.2 (0.2)^{A}	43.0 (0.6)^{B}	10.4 (0.1)^{A}	31.8 (0.4)^{C}	6.1 (0.3)^{D}	29.6 (1.0)^{E}	5.2 (0.1)^{D}	15.6 (0.2)^{F}
8	9.2 (0.1)^{A}	45.0 (0.9)^{B}	10.1 (0.2)^{A}	32.5 (1.3)^{C}	5.6 (0.2)^{D}	27.2 (1.0)^{E}	4.5 (0.2)^{D}	15.0 (0.2)^{F}
12	9.2 (1.5)^{A}	47.1 (1.2)^{B}	9.8 (0.3)^{A}	33.3 (0.7)^{C}	5.1 (0.1)^{D}	24.7 (0.9)^{E}	3.7 (0.3)^{D}	14.3 (0.1)^{F}
24	11.0 (0.6)^{A}	52.9 (2.2)^{B}	11.7 (0.4)^{A}	40.4 (0.6)^{C}	5.8 (0.1)^{D}	28.9 (0.9)^{E}	4.2 (0.0)^{D}	17.1 (0.2)^{F}
Total BCFA								
4	0.3 (0.0)^{A}	0.2 (0.0)^{B}	0.3 (0.0)^{A}	0.2 (0.0)^{B}	0.2 (0.0)^{B}	0.6 (0.0)^{C}	0.2 (0.0)^{B}	0.7 (0.0)^{D}
8	0.4 (0.0)^{A}	0.4 (0.0)^{A}	0.3 (0.0)^{B}	0.2 (0.0)^{C}	0.1 (0.0)^{D}	0.6 (0.0)^{E}	0.1 (0.0)^{D}	0.7 (0.1)^{F}
12	0.4 (0.0)^{A}	0.5 (0.0)^{B}	0.4 (0.0)^{A}	0.2 (0.0)^{C}	0.1 (0.0)^{D}	0.7 (0.0)^{E}	0.1 (0.0)^{D}	0.8 (0.1)^{F}
24	0.6 (0.0)^{A}	1.1 (0.2)^{B}	0.5 (0.0)^{A}	0.3 (0.0)^{C}	0.1 (0.0)^{D}	0.9 (0.0)^{B}	0.1 (0.0)^{D}	0.9 (0.1)^{B}
Acetate								
4	5.7 (0.1)^{A}	27.2 (0.2)^{B}	6.9 (0.2)^{C}	21.2 (0.3)^{D}	3.8 (0.2)^{E}	19.6 (0.6)^{F}	3.1 (0.0)^{E}	10.0 (0.1)^{G}
8	5.9 (0.1)^{A}	27.4 (0.5)^{B}	6.6 (0.2)^{A}	19.5 (1.1)^{C}	3.6 (0.1)^{D}	17.9 (0.6)^{E}	2.7 (0.1)^{D}	9.7 (0.1)^{F}
12	6.0 (1.1)^{A}	27.6 (0.7)^{B}	6.4 (0.1)^{A}	21.0 (0.5)^{C}	3.4 (0.1)^{D}	16.3 (0.7)^{E}	2.3 (0.2)^{D}	9.3 (0.1)^{F}
24	7.0 (0.5)^{A}	30.8 (1.3)^{B}	7.5 (0.4)^{A}	23.1 (0.3)^{C}	4.0 (0.1)^{D}	18.8 (0.6)^{E}	2.6 (0.0)^{D}	11.1 (0.1)^{F}
Propionate								
4	1.4 (0.0)^{A}	4.9 (0.1)^{B}	1.4 (0.0)^{A}	4.0 (0.0)^{C}	0.9 (0.1)^{D}	4.7 (0.2)^{B}	0.8 (0.0)^{D}	2.4 (0.0)^{E}
8	1.3 (0.0)^{A}	5.6 (0.1)^{B}	1.3 (0.0)^{C}	3.3 (0.1)^{D}	0.8 (0.0)^{AE}	4.1 (0.2)^{C}	0.7 (0.0)^{E}	2.2 (0.0)^{F}
12	1.2 (0.2)^{A}	6.2 (0.2)^{B}	1.1 (0.1)^{A}	3.2 (0.1)^{C}	0.7 (0.0)^{D}	3.5 (0.1)^{C}	0.6 (0.0)^{D}	1.9 (0.0)^{E}
24	1.4 (0.0)^{A}	6.9 (0.3)^{B}	1.4 (0.0)^{A}	3.8 (0.1)^{C}	0.8 (0.0)^{D}	4.1 (0.2)^{C}	0.7 (0.0)^{D}	2.3 (0.0)^{E}
Butyrate								
4	1.5 (0.1)^{AB}	10.0 (0.3)^{C}	1.5 (0.0)^{A}	5. 8 (0.1)^{D}	1.1 (0.1)^{BE}	4.2 (0.2)^{F}	1.0 (0.0)^{E}	2.3 (0.0)^{G}
8	1.3 (0.0)^{A}	10.4 (0.3)^{B}	1.3 (0.0)^{A}	5.7 (0.0)^{C}	0.9 (0.0)^{AD}	3.7 (0.2)^{E}	0.9 (0.0)^{D}	2.1 (0.0)^{F}
12	1.1 (0.1)^{A}	10.8 (0.4)^{B}	1.1 (0.1)^{A}	6.8 (0.1)^{C}	0.8 (0.0) ^{A}	3.2 (0.1)^{D}	0.7 (0.0)^{A}	1.8 (0.0)^{E}
24	1.2 (0.0)^{AB}	11.7 (0.5)^{C}	1.5 (0.0)^{A}	10.5 (0.3)^{E}	0.8 (0.0)^{B}	3.8 (0.1)^{D}	0.8 (0.0)^{B}	2.1 (0.0)^{E}
Valerate								
4	0.2 (0.0)^{A}	0.3 (0.0)^{B}	0.2 (0.0)^{A}	0.3 (0.0)^{B}	0.1 (0.0)^{C}	0.5 (0.0)^{D}	0.1 (0.0)^{C}	0.4 (0.0)^{E}
8	0.3 (0.0)^{A}	0.5 (0.0)^{B}	0.3 (0.0)^{A}	0.5 (0.0)^{C}	0.1 (0.0)^{D}	0.7 (0.0)^{E}	0.1 (0.0)^{D}	0.5 (0.0)^{C}
12	0.3 (0.0)^{A}	0.8 (0.0)^{B}	0.3 (0.0)^{A}	0.6 (0.0)^{C}	0.1 (0.0)^{D}	0.8 (0.0)^{B}	0.1 (0.0)^{D}	0.5 (0.0)^{E}
24	0.5 (0.0)^{A}	1.1 (0.0)^{B}	0.5 (0.0)^{A}	0.8 (0.0)^{C}	0.1 (0.0)^{D}	1.0 (0.0)^{E}	0.1 (0.0)^{D}	0.7 (0.0)^{F}
Caproate								
4	0.4 (0.0)^{A}	0.5 (0.0)^{B}	0.4 (0.0)^{A}	0.5 (0.0)^{B}	0.1 (0.0)^{C}	0.5 (0.0)^{B}	0.1 (0.0)^{C}	0.4 (0.0)^{D}
8	0.5 (0.0)^{A}	1.1 (0.0)^{B}	0.6 (0.0)^{C}	1.0 (0.0)^{D}	0.1 (0.0)^{E}	0.7 (0.0)^{F}	0.1 (0.0)^{E}	0.6 (0.0)^{C}
12	0.7 (0.1)^{A}	1.7 (0.1)^{B}	0.8 (0.1)^{AC}	1.6 (0.1)^{B}	0.1 (0.0)^{D}	0.9 (0.0)^{C}	0.1 (0.0)^{D}	0.7 (0.0)^{A}
24	0.9 (0.1)^{A}	2.4 (0.1)^{B}	0.9 (0.0)^{A}	2.2 (0.1)^{C}	0.1 (0.0)^{D}	1.1 (0.0)^{E}	0.1 (0.0)^{D}	0.9 (0.0)^{A}

*Means (SD) at 0 h for all the samples: Acetate = 1.5 (0.1); Propionate= 0.4 (0.0); Butyrate= 0.5 (0.0); Valerate= 0.1(0.0); Caproate= 0.1 (0.0); Total SCFA= 4.7 (0.12).

**Means (standard error) within row with the different capital letter superscripts are significantly different (P<0.05).

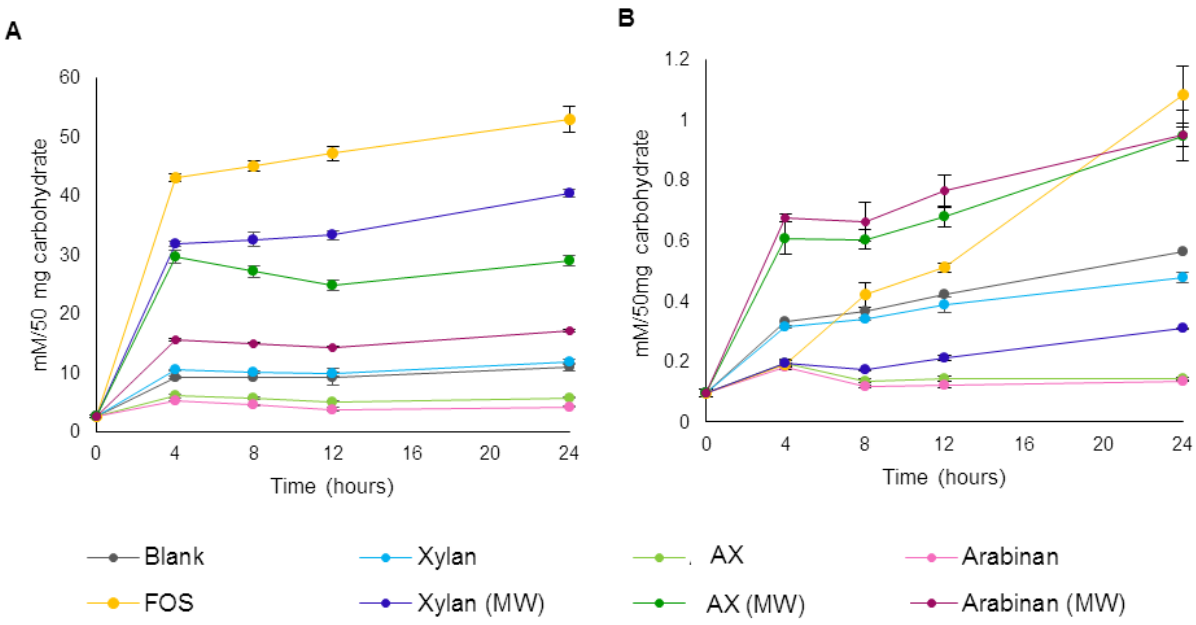

Figure 1. Total SCFA production (A) and total BCFA production (B) (mL/50mg carbohydrate) during *in vitro* fecal fermentation of native and microwave treated polysaccharides compared to FOS and to the blank.

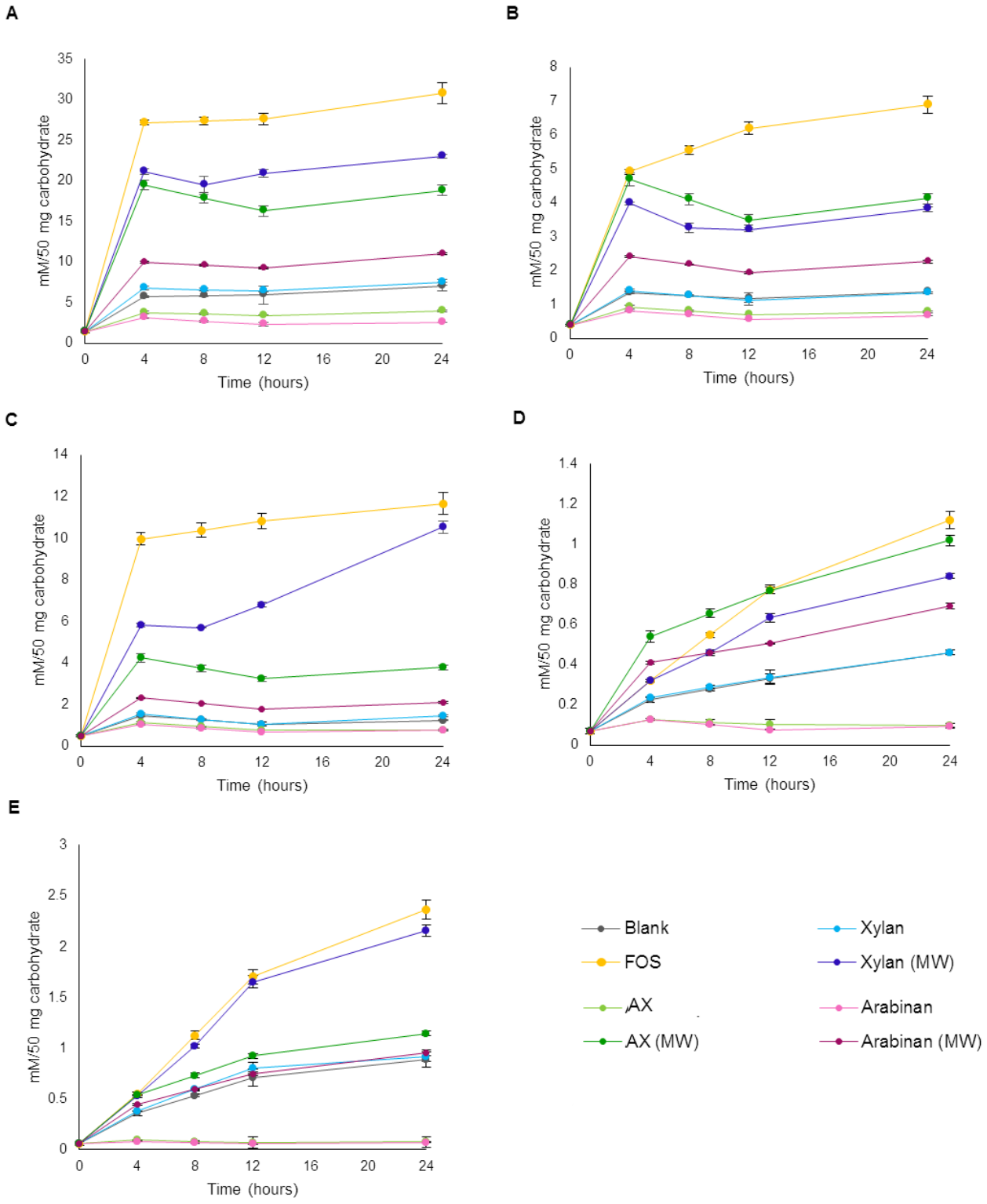

Figure 2. Acetate (A), propionate (B), butyrate (C), valerate (D) and caproate (E) production (mL/50mg carbohydrate) during *in vitro* fecal fermentation of native and microwave treated polysaccharides compared to FOS and to the blank.

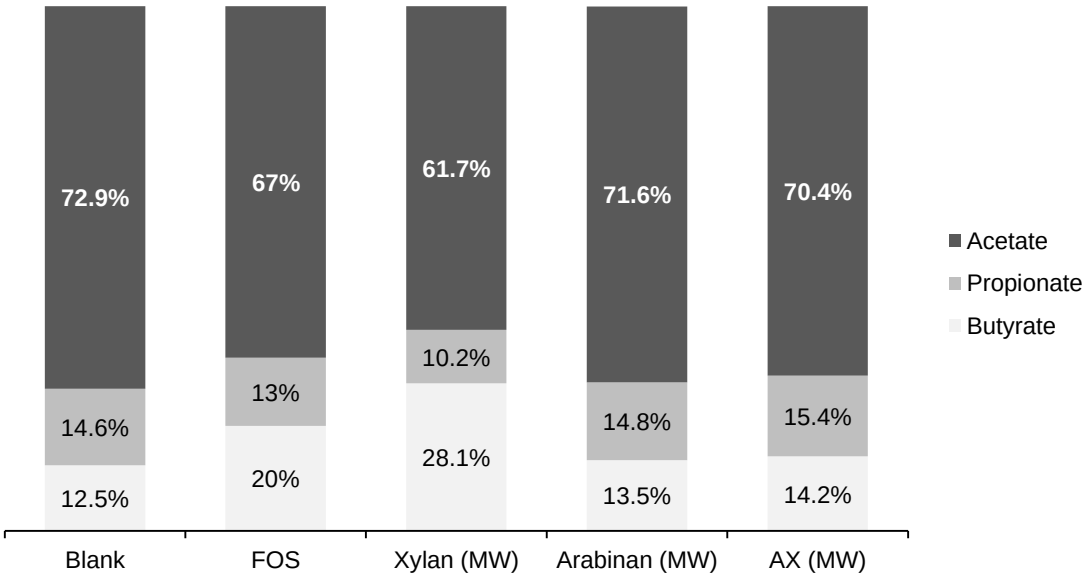

Figure 3. Acetate, butyrate and propionate percentages from total SCFA produced after 24 hour *in vitro* fecal fermentation of microwave treated polysaccharides compared to FOS and to the blank

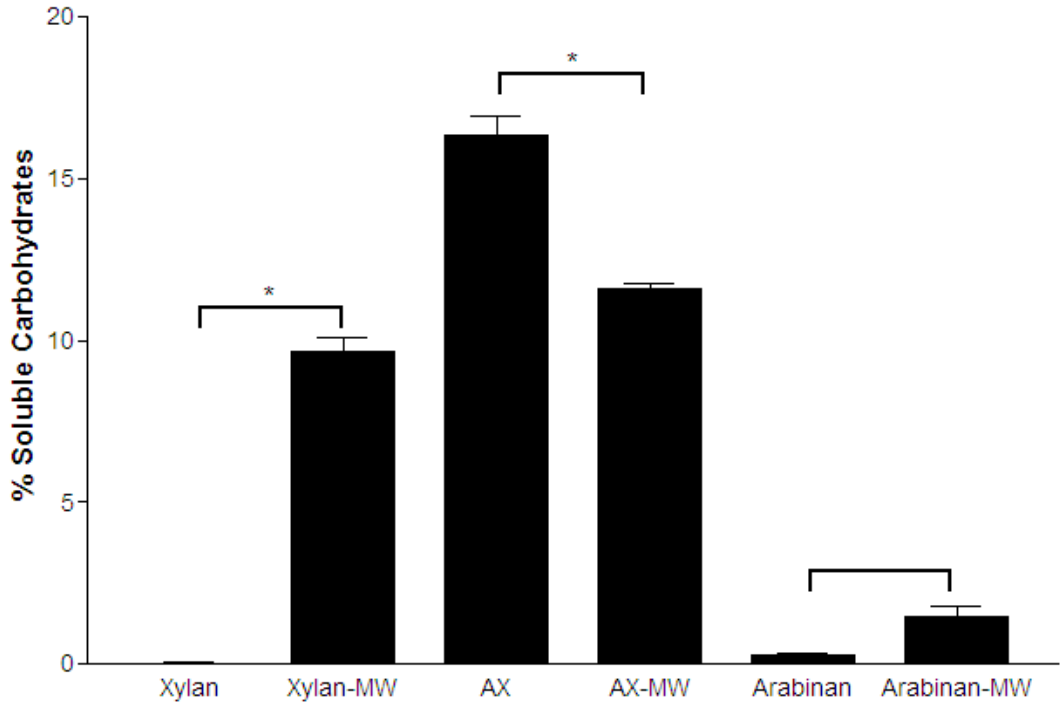

Figure 4. Soluble carbohydrate content of native and microwave treated samples expressed as percentage of total carbohydrates content.

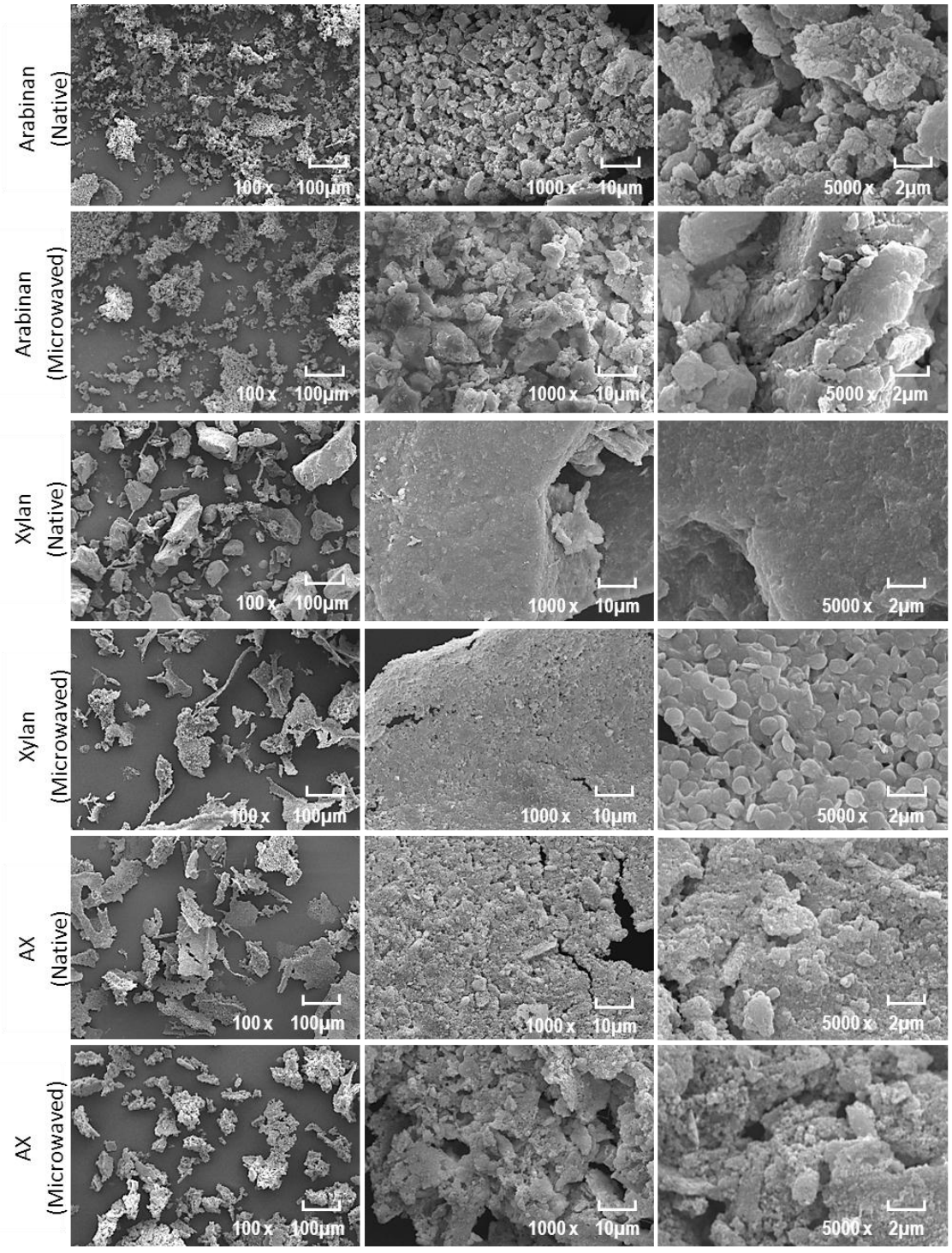

Figure 5. SEM images of samples before and after microwaving in magnifications of 100 1000 and 5000 x.

ARTIGO VI

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A pectic polysaccharide from peach palm fruits (*Bactris gasipaes*) and its fermentation profile by the human gut microbiota *in vitro*

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ABSTRACT

Depending on their chemical structure, pectic polymers may be fermented by different bacteria in the human gut and, therefore, display a different metabolite profile. We aimed to investigate the chemical structure of pectins from peach palm fruits and to evaluate their *in vitro* fecal fermentation profiles. A fraction (PW-AP) containing a linear highly methyl esterified homogalacturonan (degree of esterification of 70%) with minor portions of xylogalacturonan and type I rhamnogalacturonan was submitted to fermentation by the human gut microbiota. It produced significantly less gas than fructooligosaccharides (FOS) at all evaluated time points, and in 24 hours presented a cumulative gas production ~27% lower than FOS. Drops in pH could be observed during fermentation of both FOS and PW-AP. Moreover, the pectin was slower fermented than FOS, though with a similar production of total short chain fatty acids (SCFA) and a lower production of branched chain fatty acids. The amounts of specific SCFA differed from that of FOS, with higher production of acetate (16.2%) and propionate (6.2%) and lower production butyrate (112%). The ratio of total SCFA production to gas production was 4.0

mL/µmol and 5.5 mL/µmol for FOS and PW-AP, respectively, indicating that the latter leads to less gas formation per µmol of produced SCFA. Overall, the understanding of how pectic structures are fermented brings new insights into the fiber utilization by the human gut microbiota and its relation to biological outcomes.

Keywords: Peach palm pectins; *in vitro* fecal fermentation; short chain fatty acids.

1. Introduction

Pectic polymers are major components in the cell wall and middle lamella of fruits. Structural features of pectins are closely related to the extraction methodology, ripening stage and fruit sources (SALUNKHE & KADAM, 1995; VORAGEN, COENEN, VERHOEF, & SCHOLS, 2009). In general, pectins are formed by long linear chains of α-1,4-glycoside-linked D-galacturonic acid (homogalacturonans), that may be methyl-esterified at C-6 and *O*-acetylated at C-2 and/or C-3. The α-1,4-galacturonic acid can be linked to terminal xylose units forming xylogalacturonans. In addition, more branched and complex pectic substances are present in fruit cell walls such as type I rhamnogalacturonan, that is further branched by arabinans, galactans and/or arabinogalactans, and type II rhamnogalacturonans (RIDLEY, O'NEILL, & MOHNEN, 2001; VORAGEN et al., 2009).

Overall, pectins fall into the class of dietary fibers: polymers that cannot be depolymerized by endogenous gastrointestinal enzymes during passage through the stomach and the small intestine. In the colon, pectins are fermented more or less completely by the gut microbiota. While humans only get around 10% of daily energy requirements through fermentation of dietary fibers by intestinal microorganisms, the microbial communities and metabolites produced during dietary fibers fermentation in the colon have important consequences for host health (BESTEN et al., 2013; WONG, DE SOUZA, KENDALL, EMAM, & JENKINS, 2006)

Short chain fatty acids (SCFA) such as acetate, propionate and butyrate are the main products of dietary fiber fermentation. The rate, amount and profile of SCFA production depends on the microbiota present in the colon, the substrate structure and gut transit time (RÍOS-COVIÁN et al., 2016). After production, SCFAs can exhibit distinct biological functions in the human body. Specific SCFA may reduce the risk of developing gastrointestinal disorders, cancer, and cardiovascular disease (RÍOS-COVIÁN et al., 2016; WONG et al., 2006). Most of the produced butyrate is quickly absorbed by colonocytes and acts to nourish the colonic mucosa and in the prevention of colon cancer by promoting cell differentiation, cell-cycle arrest and apoptosis of transformed colonocytes. On the other hand, the majority of acetate and

propionate reach the portal circulation being metabolized in peripheral tissues and in the liver, respectively (RÍOS-COVIÁN et al., 2016). A reduction in acetate:propionate ratio was proposed to reduce serum lipids and possibly cardiovascular disease risk (WONG et al., 2006).

It was previously demonstrated that pectic polymers from different sources are extensively fermented by the colonic gut microbiota *in vitro* (LICHT et al., 2010; MIN et al., 2015; TITGEMEYER, BOURQUIN, FAHEY, & GARLEB, 1991). However, there is a great variety of pectic polymers from different sources containing distinct compositions, arrangements and molecular sizes that were not evaluated regarding its fermentation by the human gut microbiota.

Bactris gasipaes is a palm tree that yields two food crops with commercial potential, the fruit (peach palm) and the heart-of-palm (locally known as palmito pupunha) (CLEMENT et al., 2004). While the heart of palm is widely consumed in all regions of Brazil and has gained attention in the international market, the fruits are part of the daily diet of populations in the Northern regions of Brazil. The fruit is consumed after cooking or is used to make flours, and is of nutritional significance due to its energy and beta-carotene contents (MORA-URPÍ, WEBER, & CLEMENT, 1997).

Thus, this work aimed to characterize pectic polysaccharides from peach palm fruits (*Bactris gasipaes* Kunth) and to evaluate its fermentation profile by the human gut microbiota *in vitro*.

2. Materials and methods

2.1 Plant material

Ripe fruits of pupunha (*Bactris gasipaes*) were purchased at local market in Manaus, State of Amazonas, Brazil.

2.2 Extraction and purification of polysaccharides

Peach palm fruits were peeled and the pulp (640 g) manually removed, freeze-dried and milled (265 g). Dried pulp powder was defatted with chloroform–methanol (1:1), in order to remove lipids, pigments and other hydrophobic materials, giving 235 g of deffated pulp. The polysaccharides were then extracted with water at 100 ºC for 2 h (× 5, l L each). The aqueous extracts were clarified by centrifugation (3860*g*, 20 min at 25 ºC), pooled and concentrated under reduced pressure. The polysaccharides were precipitated with EtOH (3 vol), collected by centrifugation (3860*g*, 20 min at 4 ºC) and freeze-dried, giving fraction PW (polysaccharides from **P**each palm pulp extracted with **W**ater). In order to remove starch, PW was extensively treated with α-amylase (from *Bacillus licheniformis*, Sigma A3403) and pullulanase (from *Enterobacter aerogenes*, Sigma P2138) and further dialyzed, yielding fraction PW-AP.

Yields of polysaccharide fractions were expressed as percent based on the weight of defatted dried pulp of peach palm fruit that was submitted to extraction (235 g), whereas the moisture and lipids content were expressed as percent based on the weight of the wet peach palm fruit pulp (640 g).

2.3 Determination of homogeneity of polysaccharides and molecular weight of components

The homogeneity and average molar mass (M_w) of PW-AP was determined by gel permeation chromatography (GPC), using a refractometer as detection equipment. Four columns were used in series, with exclusion sizes of 7×10^6 Da (Ultrahydrogel 2000, Waters), 4×10^5 Da (Ultrahydrogel 500, Waters), 8×10^4 Da (Ultrahydrogel 250, Waters) and 5×10^3 Da (Ultrahydrogel 120, Waters). The eluent was 0.1 M aq. $NaNO_2$ containing 200 ppm aq. NaN_3 at 0.6 ml/min. The samples, previously filtered through a membrane (0.22 µm, Millipore), were injected at a concentration of 1 mg/ml. To obtain the molecular weight, standard dextrans (487

kDa, 266 kDa, 124 kDa, 72.2 kDa, 40.2 kDa, 17.2 kDa and 9.4 kDa, from Sigma) were employed to obtain the calibration curve. The molecular weight of the sample was calculated according to the calibration curve.

2.4 Sugar composition

Neutral monosaccharide components of the polysaccharides and their ratio were determined by hydrolysis with 2 M TFA for 8 h at 100 ºC, followed by conversion to alditol acetates by successive $NaBH_4$ reduction and acetylation with Ac_2O-pyridine (1:1, v/v, 1mL) at room temperature for 14 h, and the resulting alditol acetates extracted with $CHCl_3$. These were analyzed by GC–MS using a Varian Saturn 2000R model 3800 gas chromatograph linked to a Varian Ion-Trap 2000R mass spectrometer, with He as the carrier gas. A fused-silica capillary column (30 m x 0.25 mm) coated with DB-225MS (Durabond) was used, held at 50 ºC during injection for 1 min, then programmed at 40 ºC/min to 220 ºC and held at this constant temperature for 19.75 min.

Uronic acid content was determined spectrophotometrically using the *m*-hydroxybiphenyl method (FILISETTI-COZZI & CARPITA, 1991).

2.5 Methylation analysis of polysaccharide

Fraction PW-AP was *O*-methylated as described by Ciucanu and Kerek (CIUCANU & KEREK, 1984). The per-*O*-methylated polysaccharide was further submitted to methanolysis in 3% HCl–MeOH (80 °C, 2 h) followed by hydrolysis with H_2SO_4 (0.5M, 14 h) and neutralization with $BaCO_3$. The material was then reduced and acetylated as described above for monosaccharide composition, except that $NaBD_4$ was used for reduction. The resultant partially *O*-methylated alditol acetates were analyzed with a GC-MS. For separation, a 30 m x 0.25 mm i.d. capillary column of DB-225 was held at 50 ºC during injection for 1 min, then programmed at 40

ºC/min to 210 ºC and held at this temperature for 31 min. Typical electron impact breakdown profiles and retention times of partially *O*-methylated alditol acetates were used for identification (SASSAKI, GORIN, SOUZA, CZELUSNIAK, & IACOMINI, 2005).

2.6 Nuclear magnetic resonance (NMR) spectroscopy

^{13}C NMR and $^{1}H/^{13}C$ HSQC-NMR spectra were acquired at 70°C on a Bruker AVANCE III 400 NMR spectrometer (Bruker Corporation, Billerica – MA, USA), operating at 9.5 T, and observing ^{13}C at 100.61 MHz; equipped with a 5-mm multinuclear inverse detection probe with z-gradient. All ^{13}C and $^{1}H/^{13}C$ HSQC -NMR chemical shifts were expressed in ppm relative to the CH_3 signal from acetone at δ 30.2/2.22 as the internal reference. All pulse programs were supplied by Bruker.

The degree of methyl esterification (DE) was determined by ^{1}H NMR spectroscopy according Grasdalen, Bakoy & Larsen (1988). Briefly, the fraction was deuterium-exchanged three times by freeze-drying with D_2O solutions, and finally dissolved in D_2O and transferred into a 5-mm NMR tube. The ^{1}H NMR spectrum was acquired at 70 °C with 256 scans, at pD 5.0, on a Bruker AVANCE III 400 NMR spectrometer, operating at 9.5 T, observing ^{1}H at 400.13 MHz, and equipped with a 5-mm multinuclear inverse detection probe with z-gradient.

2.7 *In vitro* fecal fermentation

Batch fecal fermentation was performed as the methodology of Lebet et al. (1998) with some minor modifications (ROSE et al., 2010). The total carbohydrate content in the sample was determined by the phenol-sulphuric acid method (DUBOIS, GILLES, HAMILTON, REBERS, & SMITH, 1956). Each substrate (50 mg equivalent carbohydrate) was weighed in 3 test tubes for each time point such that all

the analysis occurred in triplicate. Hydration of samples was performed by adding 4 mL of carbonate-phosphate buffer pH 6.8 ± 0.1 to each tube. Fructooligosaccharides (FOS - No. F8052, Sigma-Aldrich Inc., St. Louis, Mo., USA) and tubes without any added carbohydrate were used as positive and negative controls, respectively.

Fecal samples were obtained from 3 healthy volunteers who were on their routine diet and had not taken antibiotics within the previous 6 months. Fecal samples were collected in plastic bags that were sealed after escaping the air, transported on ice, and immediately placed inside an anaerobic chamber (10% H_2, 5% CO_2, and 85% N_2; BactronEZ, SHEL LAB, Cornelius, OR) where all further procedures were performed within 2 hours after collection. Fecal samples were pooled together and the fecal slurry prepared by homogenization with carbonate-phosphate buffer pH 6.8 ± 0.1 in a ratio of 1:3 (w/v) and further strained through 4 layers of cheese cloth. The filtrate (1 mL) was then inoculated with the hydrated carbohydrate sample and the controls. Tubes were sealed and incubated at 37 ºC in a shaking water bath. Immediately after incubation and after 4, 8, 12, and 24 h of fermentation, assigned tubes were removed from the water bath, and total gas volume was measured. To terminate microbial activity, copper sulphate solution (400 µL of 2.75 mg/mL) was added and pH of samples recorded. Aliquots (400 µL) were combined with 100 µL of a mixture containing 50 mM 4-methyl-valeric acid (No. 277827- 5G, Sigma-Aldrich Inc., St. Louis, Mo., USA), 5% meta-phosphoric acid, and copper sulphate (1.56 mg/mL); and mixed with a vortex mixer and stored at - 40 ºC until used for SCFAs analysis. To quantify SCFA content, frozen fermentation residues were thawed and centrifuged at 13,000 rpm for 10 min. An aliquot (0.2 µL) was injected into a GC-FID 7890A (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a fused silica capillary column (NukolTM , Supelco No. 40369-03A, Bellefonte, Pa.,USA). The initial oven temperature was held at 50 °C for 2 min, ramped to 70 °C at a rate of 10 °C/min, to 85 °C at a rate of 3 °C/min, to 110 °C at a rate of 5 °C/min, to 290 °C at a rate of 30 °C/min, and finally held at 290 °C for 8 min. Helium was used as a carrier gas at a constant flow rate of 1 mL/min through the column.

2.8 Statistical analysis

Statistical differences among data were determined using GraphPad Prism software package version 7.0 (GraphPad Software, Inc., California, U.S.A.). Differences in gas, pH, and each SCFA produced at each time point among substrates were analyzed using repeated measures analysis of variance and differences between least square means were calculated using Tukey's multiple comparisons test.

3. Results and discussion

3.1 Structural characterization of pectic polysaccharides

The fresh pulp of peach palm fruits (640 g), with approximately 58% moisture content, was freeze-dried. The dried pulp powder (265 g) was then defatted yielding 0.5% of lipids. The residue was further submitted to successive extractions with hot water and polysaccharides were precipitated with 3 vol ethanol to give rise to fraction PW (29% yield).

Monosaccharide analysis showed that glucose represented 91.5% of all neutral sugars in PW (Table 1). Likewise, its ^{13}C NMR spectrum (data not shown) presented six main signals of 1,4-linked α-D-glucose at δ 99.7 (C-1), 71.7 (C-2), 73.4 (C-3), 77.5 (C-4), 71.4 (C-5) and 60.7 (C-6), indicating the presence of starch. In order to remove it, PW was extensively treated with α-amylase and pullulanase, yielding fraction PW-AP (1.2% yield).

Fraction PW-AP, was analyzed through HPSEC and presented a homogeneous profile (Figure 1) with an average molecular weight (*Mw*) of 17 kDa. Monosaccharide analysis showed high uronic acid content, followed by small amounts of arabinose, xylose, rhamnose and galactose (Table 1).

The ^{13}C- and $^{1}H/^{13}C$ HSQC -NMR (^{1}H anomeric region) spectra of fraction PW-AP are shown in Figure 2A and B. The more intense signal in the anomeric region of the spectra, at δ 100.0/4.97 and δ 99.2/5.14, could be assigned to anomeric carbons of esterified and unesterified units of α-D-Gal*p*A, respectively. The C-6 signal from esterified carbonyl carbons could be seen at δ 170.6 and from methyl carbons of esterified carbonyls in Gal*p*A units at δ 52.8. The remaining carbons of the α-D-Gal*p*A ring were seen at δ 78.6/4.46 (*O*-substituted C-4), δ 70.5/5.11-5.05 (C-5 of esterified units), δ 71.6/4.70 (C-5 of unesterified units), δ 68.3 (C-2 and C-3 overlapped). The degree of methyl esterification (DM) calculated by ^{1}H NMR was of 70%. These data show the presence of a high methyl-esterified (HM) homogalacturonan in PW-AP. Moreover, signals of lower intensity could also be noted in the anomeric region of PW-AP ^{13}C NMR spectrum. Anomeric signals at δ 109.1/5.26 and δ 107.5/5.08 were assigned to α-arabinofuranose and at δ 103.2/4.51 to β-galactopyranose residues. The anomeric signal at δ 102.4/4.51 was tentatively assigned to C-1 of xylopyranose residues, once these were also found in monosaccharide analysis.

Methylation data (Table 2) corroborates with ^{13}C NMR findings. The main derivative after PW carboxyreduction and methylation was 2,3,6-Me_3-Gal-ol acetate, that arises mostly from (1→4)-linked Gal*p*A units from the homogalacturonan structure. The minor presence of the derivatives 2,3,4-Me_3-Xyl-ol-acetate and 2,3-Me_2-Xyl-ol-acetate suggest that some Gal*p*A units of the homogalacturonan could be substituted by terminal and 4-*O*-substituted xylosyl units, respectively, possibly trough 3-*O*-substituted Gal*p*A units, once the derivative 2,6-Me_2-Gal-ol-acetate was found. In pectic polymers, regions containing Gal*p*A substituted by xylose at *O*-3 position, are known as xylogalacturonans (VORAGEN et al., 2009). Moreover, small amounts of the rhamnose derivatives 3,4-Me_2- and 3-Me- demonstrated the presence of (1→2)- and (1→2,4)-linked-Rha*p* units. These are characteristic of type I rhamnogalacturonans, commonly found interspersed with Gal*p*A in pectic polymers, usually further branched by neutral side chains (VORAGEN et al., 2009). Thus arabinose derivatives such as 2,3,5-Me_3-Ara-ol-acetate and 2,3-Me_2-Ara-ol-acetate and galactose derivatives such as 2,3-Me_2 -Gal-ol acetate and possibly small portions of the 2,3,6-Me_3- and 2,3,4,6-Me_4 -Gal-ol acetate are present as a part of the type I rhamnogalacturonan side chains. In summary, fraction PW-AP was composed

mainly by a linear highly methyl esterified homogalacturonan with minor portions of xylogalacturonan and type I rhamnogalacturonan.

3.2 Fermentation profile of PW-AP by the human gut microbiota *in vitro*

Fraction PW-AP (93.8% total carbohydrates) was submitted to *in vitro* fecal fermentation and the profiles of generated metabolites (gas, pH and SCFA) were analyzed in 4, 8, 12 and 24 hours of fermentation. Table 3 shows the amount of gas produced and pH alterations during *in vitro* fermentation. Fraction PW-AP produced significantly less gas than FOS at all evaluated time points, and in 24 hours presented a cumulative gas production ~27% lower than FOS. Drops in pH could be observed during fermentation for FOS and PW-AP, especially in the initial 4 hours, while the blank pH remained almost unchanged in all time points. Fructooligosaccharides led to a wider gas production and reduction in pH if compared to PW-AP (Table 3), however, the total SCFA produced for both samples were not significantly different (Table 4). Although gas production is inherent to the fermentation process that results in the production of short chain fatty acids, it's also responsible for the adverse effects of prebiotics in humans, such as abdominal discomfort. Thus, the ratio of total SCFA production to gas production could be used to estimate tolerability when administrated *in vivo*. These were of 4.0 mL/µmol and 5.5 mL/µmol for FOS and PW-AP, respectively, indicating that the latter leads to less gas formation per µmol of produced SCFA. Similarly to what was found for the pH, most of the SCFA production occurred within the first 4 hours of fermentation for both FOS and PW-AP. Yet, PW-AP produced 36% of the total SCFA between 4-24 hours, while FOS produced only 17% in the same evaluated period indicating that PW-AP was slower fermented than FOS (Table 4).

Therefore, the fermentation pattern of the HM pectin in fraction PW corroborates previous findings of *in vitro* and *in vivo* studies that demonstrated that higher degrees of methylation in pectic polymers lead to a slower fermentation profile (DONGOWSKI, LORENZ, & PROLL, 2002; TIAN et al., 2016).

Regarding specific SCFAs, acetate and propionate production were higher for PW-AP (16.2% and 6.2%, respectively) than for FOS after 24 hours of fermentation. FOS led to a higher butyrate production, around 112% more than PW-AP (Table 3). Indeed, other HM pectic polymers, have shown to be specifically acetogenic with a low butyrate production (GULFI, ARRIGONI, & AMADÒ, 2005; JONATHAN et al., 2012). Jonathan et al. (2012) found that the fermentation of a HM pectin using human fecal inocula resulted in a 80:14:7 acetate: propionate:butyrate ratio, whereas in our study a similar proportion of 75:15:10 was found.

Concerning to the production of branched chain fatty acids (BCFA), PW-AP and the blank presented a similar low production, while FOS led to a BCFA production 85.2% higher than PW-AP after 24 hours of fermentation. It's noteworthy however, that BCFA production with FOS was still small and represented only ~3% from the total SCFA (Table 3).

In summary, our data shows that PW-AP fermentation leads to a similar production of total SCFA than FOS, with a higher production of acetate and lower production of BCFA. Acetate is often used to monitor colonic events because it is the primary SCFA produced in the human gut and reaches the highest plasma concentrations (BESTEN et al., 2013; COOK & SELLIN, 1998). Moreover, pH decreases generated by acetate production are related to a beneficial influence on the composition of gut microbiota and prevents the proliferation of harmful species and growth of pathogenic bacteria. Finally, some bacteria in the human colon could convert part of the acetate produced into other beneficial metabolites to the host, such as butyrate (DIEZ-GONZALEZ, BOND, JENNINGS, & RUSSELL, 1999; DUNCAN, HOLD, BARCENILLA, STEWART, & FLINT, 2002; RÍOS-COVIÁN et al., 2016).

It's well known that fine chemical structures within a polymer may direct its fermentability in the human gut. Different sources of pectin such as soy, citrus, sugar beet, lemon peel were previously shown to influence fermentation by colonic bacteria (DONGOWSKI et al., 2002; MIN et al., 2015; TIAN et al., 2016; TITGEMEYER et al., 1991). Overall, this work describes structural features of pectin from peach palm fruit as well as its fermentation profile and expands our understanding about how pectic structures are fermented and influence metabolites generated in that process.

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Figure 1. GPC elution profile of fraction PW-AP, obtained from the pulp of peach palm fruits (refractive index detector). Peak retention times of dextran standards of molecular weight 487 kDa, 266 kDa, 124 kDa, 72.2 kDa, 40.2 kDa, 17.2 kDa and 9.4 kDa, respectively, are marked with arrows (left to right).

Figure 2. ^{13}C-NMR (A) and ^{1}H/^{13}C HSQC-NMR (B) spectra of PW-AP in D_2O at 70ºC, (chemical shifts are expressed as δ PPM) obtained from the pulp of peach palm fruits (*Bactris gasipaes*).

Table 1 Monosaccharide composition of fractions obtained from water extraction of peach palm pulp (*Bactris gasipaes*).

Fractions	**Monossaccharide composition (%)[a]**					
	Rha	**Ara**	**Xyl**	**Gal**	**Glc**	**Uronic acid[b]**
PW	tr[c]	4.1	2.6	tr[c]	91.5	n.d.[d]
PW-AP	3.9	14.5	6.5	3.6	-	71.5

[a] % of peak area relative to total peak areas, determined by GC–MS

[b] Determined spectrophotometrically using the *m*-hydroxybiphenyl method (Tullia M. C. C. Filisetti-Cozzi & Nicholas C. Carpita, 1991).

[c] Trace amounts.

[d] Not determined.

Table 2 Linkage types based on analysis of partially *O*-methyl alditol acetates obtained from carboxy-reduced fraction PW-AP from peach palm fruits

Partially *O*-methylalditol acetate	**PW-AP[b]**	**Linkage type[c]**
2,3,5-Me_3-Ara[a]	5.0	Ara*f*-(1→
2,3-Me_2-Ara	4.9	→5)-Ara*f*-(1→
3-Me-Ara	4.6	→2,5)-Ara*f*-(1→
2,3,4 –Me_3-Xyl	4.8	Xyl*p*-(1→
2,3 –Me_2-Xyl	2.2	→4)-Xyl*p*-(1→
3,4 –Me_2-Rha	1.2	→2)-Rha*p*-(1→
3 –Me-Rha	2.8	→2,4)-Rha*p*-(1→
2,3,4,6-Me_4-Gal	7.8	Gal*p*-(1→
2,3,6-Me_3-Gal	58.1	→4)-Gal*p*-(1→
2,6-Me_2-Gal	4.1	→3,4)-Gal*p*-(1→
2,3-Me_2-Gal	3.6	→4,6)-Gal*p*-(1→

[a] 2,3,5-Me_3-Ara = 2,3,5-tri-*O*-Methylarabinitolacetate, etc.

[b] % of peak area of *O*-methylalditol acetates relative to total area, determined by GC-MS.

[c] Based on derived *O*-methylalditol acetates.

Table 3 Gas produced (mL/50mg carbohydrate) and pH changes during *in vitro* fecal fermentation of peach palm polysaccharides compared to FOS and Blank

Time (h)	Blank	FOS	PW-AP
Gas			
4	2.8 (0.2)^{A}	12.1(0.3)^{B}	9.6 (0.2)^{C}
8	3.0 (0.0)^{A}	15.0 (0.0)^{B}	11.3 (2.2)^{C}
12	3.6 (0.2)^{A}	15.7 (0.2)^{B}	12.0 (0.0)^{C}
24	3.5 (0.4)^{A}	17.0 (0.1)^{B}	12.4 (0.2)^{C}
pH			
0	7.4 (0.03)^{A}	7.4 (0.03)^{B}	7.4 (0.03)^{C}
4	7.5 (0.01)^{A}	6.5 (0.05)^{B}	6.8 (0.02)^{C}
8	7.5 (0.01)^{A}	6.5 (0.03)^{B}	6.9 (0.03)^{C}
12	7.4 (0.00)^{A}	6.5 (0.05)^{B}	6.8 (0.01)^{C}
24	7.5 (0.02)^{A}	6.6 (0.01)^{B}	6.9 (0.01)^{C}

*Measurements at 0 h for all the samples: gas= 2.0 mL; pH= 7.43.

**Means (standard error) within row with the different capital letter superscripts are significantly different ($P<0.05$).

Table 4 Short chain fatty acid and branched chain fatty acid production (µmol/50 mg carbohydrate) during *in vitro* fecal fermentation of peach palm polysaccharides compared to FOS and Blank

Metabolites **Time (h)**	**Blank**	**FOS**	**PW-AP**
Acetate			
4	5.8 (0.7)^{A}	37.3 (1.3)^{B}	34.7 (0.3)^{C}
8	8.3 (0.1)^{A}	39.0 (0.9)^{B}	47.0 (1.0)^{C}
12	8.1 (0.2)^{A}	43.1 (0.4)^{B}	47.7 (3.6)^{C}
24	11.1 (0.5)^{A}	43.9 (1.4)^{B}	51.4 (1.0)^{C}
Propionate			
4	1.8 (0.2)^{A}	8.4 (0.1)^{B}	6.6 (0.0)^{C}
8	2.1 (0.1)^{A}	9.1 (0.1)^{B}	8.5 (0.2)^{C}
12	2.1 (0.1)^{A}	9.9 (0.1)^{B}	9.1 (0.3)^{C}
24	2.9 (0.1)^{A}	9.7 (0.4)^{B}	10.3 (0.2)^{C}
Butyrate			
4	1.5 (0.1)^{A}	10.9 (0.3)^{B}	4.1 (0.2)^{C}
8	1.7 (0.1)^{A}	13.7 (0.4)^{B}	5.3 (0.2)^{C}
12	1.7 (0.1)^{A}	14.5 (0.1)^{B}	5.8 (0.1)^{C}
24	2.0 (0.2)^{A}	14.0 (1.0)^{B}	6.6 (0.1)^{C}
Total SCFA			
4	9.2 (1.0)^{A}	56.7 (1.6)^{B}	45.4 (0.4)^{C}
8	12.1 (0.2)^{A}	61.8 (1.4)^{B}	60.8 (1.4)^{B}
12	12.0 (0.4)^{A}	67.5 (0.6)^{B}	62.5 (4.0)^{C}
24	16.0 (0.7)^{A}	67.6 (2.9)^{B}	68.4 (1.3)^{B}
Total BCFA			
4	0.49 (0.04)^{A}	0.32 (0.04)^{B}	0.22 (0.01)^{C}
8	0.53 (0.03)^{A}	0.85 (0.05)^{B}	0.39 (0.01)^{C}
12	0.62 (0.06)^{A}	1.13 (0.05)^{B}	0.52 (0.04)^{C}
24	0.76 (0.00)^{A}	1.50 (0.09)^{B}	0.81 (0.01)^{A}

*Metabolites at 0 h for all the samples: Acetate = 2.8 (±0.08); Propionate= 0.9 (±0.02); Butyrate= 0.9 (±0.02); Total SCFA= 4.7 (±0.12); Total BCFA= 0.2 (±0.01). **Means (standard error) within row with the different capital letter superscripts are significantly different ($P<0.05$).

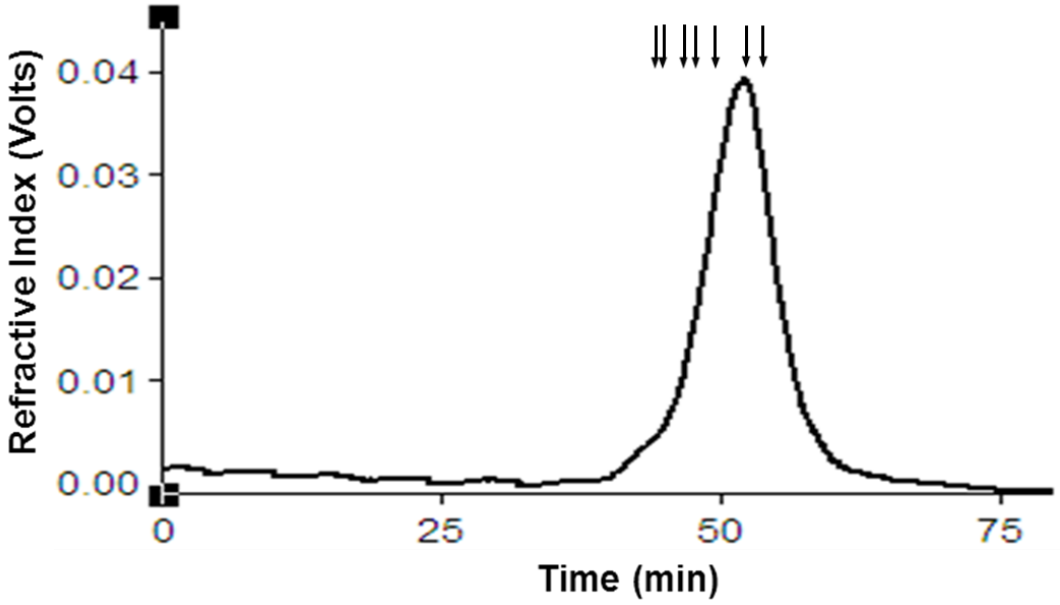

Figure 1

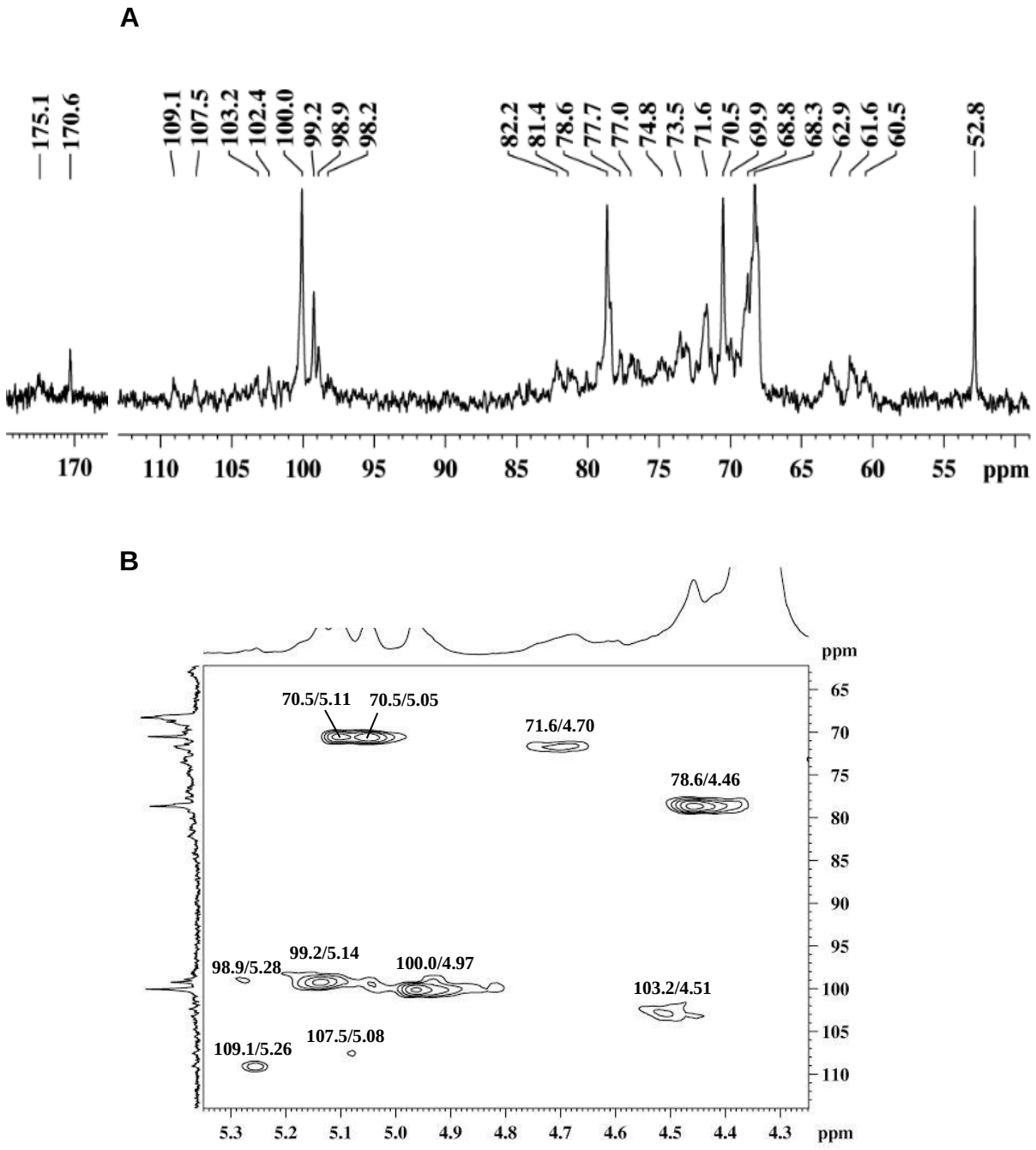
A
175.1
170.6
109.1
107.5
103.2
102.4
100.0
99.2
98.9
98.2
82.2
81.4
78.6
77.7
77.0
74.8
73.5
71.6
70.5
69.9
68.8
68.3
62.9
61.6
60.5
52.8
170
110
105
100
95
90
85
80
75
70
65
60
55
ppm
B
ppm
70.5/5.11
70.5/5.05
71.6/4.70
78.6/4.46
98.9/5.28
99.2/5.14
100.0/4.97
103.2/4.51
109.1/5.26
107.5/5.08
65
70
75
80
85
90
95
100
105
110
5.3
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
ppm

Figure 2

CONCLUSÕES

Dois aspectos principais foram abordados ao longo deste trabalho: 1) avaliação da estrutura química de polissacarídeos do buriti, tucumã, açaí e pupunha com enfoque majoriátrio do ponto de vista taxonômico e 2) o perfil de fermentação *in vitro* pela microbiota intestinal humana dos polímeros obtidos para avaliação de seu potencial prébiótico.

De maneira geral, hemiceluloses foram encontradas como componente principal no tucumã (galactoarabinoxilana> arabinana> glucuronoxilana> xiloglucanas) e açaí (xilana linear). Este último também apresentou menores porções de polissacarídeos pécticos (homogalacturonana e arabinogalactana do tipo II). O buriti apresentou polissacarídeos pécticos ricos em arabinose em quantidade similar ao seu conteúdo de hemiceluloses. Uma pectina majoritariamente linear também foi obtida a partir da pupunha, entretanto, representou apenas uma pequena parte do total de polissacarídeos extraídos, e, portanto, a avaliação total de seus componentes deve ser conduzida para maiores conclusões do ponto de vista taxonômico. Dessa forma, a análise da estrutura química dos polissacarídeos do buriti, tucumã, açaí e pupunha indica maior variedade de polímeros na sua parede estrutural primária do que anteriormente previso para membros da família Arecaceae.

Com relação ao potencial prebiótico dos polissacarídeos obtidos, polímeros com baixa solubilidade como a xilana (extraída do açaí), arabinoxilanas e arabinana (extraídas do tucumã) apresentaram aumento significativo do seus perfil de fermentação *in vitro* após tratamento com microondas. Alterações nas suas estruturas tridimensionais e perfil de solubilidade causadas pelo tratamento com microondas foram observadas e podem estar relacionadas ao aumento de sua fermentação *in vitro*. A fermentação dos polissacarídeos extraídos da pupunha também foi avaliada e levou a produção de ácidos graxos de cadeia curta totais similar a FOS, entretanto com diferentes proporções de cada um dos AGCC. A compreensão de como diferentes estruturas químicas influenciam os metabólitos

gerados durante a fermentação de polissacarídeos solúveis e insolúveis aqui realizada, traz novas perspectivas para o desenvolvimento e aplicação de prebióticos.

Por fim, ressalta-se que a xilana linear encontrada no açaí é interessante por outros pontos de vista. Sua obtenção em alto rendimento é de relevância industrial e na área biomédica. O açaí representa a maior fonte deste tipo de polímero já relatada em frutos.

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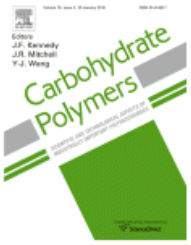

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Author: Thaisa Moro Cantu-Jungles,Carolina Pierobom de Almeida,Marcello Iacomini,Thales R. Cipriani,Lucimara M.C. Cordeiro

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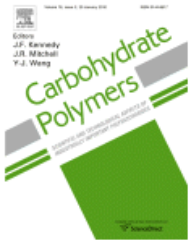

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Author: Thaisa Moro Cantu-Jungles,Marcello Iacomini,Thales R. Cipriani,Lucimara M.C. Cordeiro

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