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1 **Highlights**

- 2 Covalently crosslinked arabinoxylans (AX) gels with different structure were obtained
- 3 AX gels were biodegradable by mixture of two *Bifidobacterium*
- 4 Changes in microstructure of AX gels allowed different degradation profiles
- 5 AX gels could be used as microflora-activated system for colon delivery

***In vitro* degradation of covalently cross-linked arabinoxylan hydrogels by
Bifidobacteria**

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Abstract

Arabinoxylan gels with different cross-linking densities, swelling ratios, and rheological properties were obtained by increasing the concentration of arabinoxylan from 4 to 6% (w/v) during oxidative gelation by laccase. The degradation of these covalently cross-linked

gels by a mixture of two *Bifidobacterium* strains (*Bifidobacterium longum* and *Bifidobacterium adolescentis*) was investigated. The kinetics of the evolution of structural morphology of the arabinoxylan gel, the carbohydrate utilization profiles and the bacterial production of short-acid fatty acid (SCFA) were measured. Scanning electron microscopy analysis of the degraded gels showed multiple cavity structures resulting from the bacterial action. The total SCFA decreased when the degree of cross-linking increased in the gels. A slower fermentation of arabinoxylan chains was obtained for arabinoxylan gels with more dense network structures. These results suggest that the differences in the structural features and properties studied in this work affect the degradation time of the arabinoxylan gels.

Keywords: cross-linked arabinoxylans, gels, biodegradability, bifidobacteria, fermentation

1. Introduction

Polysaccharide-based hydrogels have been extensively studied for their potential as colon-specific drug delivery systems due to their chemical and three-dimensional structures, biocompatibility properties and swelling (Peppas, 1997; Sinha & Kumria, 2001). However, most of these hydrogels are susceptible to changes according to the low gastrointestinal pH or the duration of transit time, thus leading to premature drug release. For these reasons, some polysaccharides such chitosan, alginate and pectin have been chemically modified or cross-linked or combined with hydrophobic polymers for use in the development of systems that are specifically biodegradable by colonic bacteria (Brøndsted, Andersen, & Hovgaard, 1998; Chamblin, Dupuis, Champion, Voilley, & Pourcelot, 2006; Tozaki et al.,

1997). Covalent cross-linking has been used to enhance the mechanical strength and chemical stability of the hydrogels. However, the chemical cross-linking agents used are often toxic compounds that require harsh reaction conditions. Another alternative is to use polysaccharides, which can form covalent hydrogels via enzymatic cross-linking. Biological enzymes can catalyze polysaccharide cross-linking into gels via strong covalent bonds under mild conditions in the absence of organic solvents (Figueroa-Espinoza et al., 1999; Micard & Thibault, 1999). Unlike most polysaccharides, arabinoxylans (AX) can form covalent hydrogels via enzymatic cross-linking of ferulic acid esterified to the polysaccharide (Izydorczyk & Biliaderi, 1995; Vansteenkiste, Babot, Rouau, & Micard, 2004). Covalently cross-linked AX gels are thermo-irreversible (Carvajal-Millan, Guigliarelli, Belle, Rouau, & Micard, 2005), generally present an absence of pH or electrolyte susceptibility and exhibit no syneresis after long periods of storage (Izydorczyk & Biliaderis, 1995).

AX are non-starch polysaccharides that resist digestion and absorption in the human small intestine and are fermented in the large intestine by microbiota in the colon, especially *Bacteroides* and *Bifidobacterium* (Hopkins et al., 2003; Hughes et al., 2007). The basic structure of AX consists of a linear backbone chain of xylose units containing arabinose substituents attached through O-2 and/or O-3 (Izydorczyk & Biliaderis, 1995). Some of the arabinose residues are ester-linked to ferulic acid (FA) through (O)-5 (Smith & Hartley, 1983). FA oxidation by enzymatic free radical generating agents (laccase or peroxidase/H₂O₂) allows for the coupling of AX chains through the formation of FA dimers and trimers (di-FA, tri-FA) as covalent cross-linking structures (Carvajal-Millan, Landillon, et al., 2005; Izydorczyk & Biliaderis, 1995). Recent studies have demonstrated that AX gels formed via oxidative cross-linking can be used for the controlled release of model proteins (Carvajal-

87 Millan, Guilbert, Doublier, & Micard, 2006), methylxanthine (Iravani, Fitchett, & Georget,
88 2011), diclofenac sodium (Iqbal, Akbar, Hussain, Saghir, & Sher, 2011), lycopene
89 (Hernández-Espinoza, Piñón-Muñiz, Rascón-Chu, Santana-Rodríguez, & Carvajal-Millan,
90 2012) and probiotic bacteria (Morales-Ortega et al., 2014).

91 The degradation of the AX structure results from the activity of different bacterial enzymes,
92 which mainly include endo-xylanases, beta-xylosidases, reducing end xylose-releasing exo-
93 oligoxylanases and alpha-L-arabinofuranosidases (Pollet et al., 2012). The mechanism of
94 AX degradation depends on the degree of polymerization, crosslinking and branching
95 present in the AX structure (Hughes et al., 2007; Rose, Patterson, & Hamaker, 2010).
96 Notably, bifidobacteria comprise up to 25% of the cultivable gut microflora, with
97 *Bifidobacterium longum* and *Bifidobacterium adolescentis* predominating in adults.
98 Mixtures of these strains have been associated with the complete fermentation of low
99 molecular weight arabinoxylans (Pastell, Westermann, Meyer, Tuomainen, & Tenkanen,
100 2009). Though the fermentation properties of arabinoxylans have been researched
101 extensively, only a few studies have reported the degradation of the covalently cross-linked
102 arabinoxylan network, and these studies did not report the structural features, cross-linking
103 density and mechanical properties of the gel (Hopkins et al., 2003). These structural
104 parameters of AX matrix gel could influence the ability of colonic microflora to degrade
105 polysaccharides. In the present study, the ability of a mixture of *Bifidobacterium longum*
106 and *Bifidobacterium adolescentis* to degrade covalent arabinoxylan hydrogels with
107 different swelling and structural parameters was examined.

108

109 2. Experimental

110 2.1. Materials

111 Maize bran arabinoxylans (MBAX) were obtained and characterized as previously reported
112 by Martínez-López et al. (2012). MBAX contained 85% dry basis (d.b.) of pure AX,
113 contained ferulic acid (FA), di-FA, and tri-FA contents of 0.25, 0.14, and 0.07 µg/mg of
114 MBAX, respectively, and an A/X ratio of 0.72. Laccase (benzenediol: oxygen
115 oxidoreductase, E.C.1.10.3.2) was isolated from *Trametes versicolor* and all other chemical
116 products were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

117 2.2. Preparation of MBAX gels

118 MBAX solutions at 4% (MBAX-4) and 6% (MBAX-6) (w/v) were prepared in 0.1 M
119 acetate buffer (pH 5.5). The buffer was filtered through a 0.2 µm filter to prevent microbial
120 contamination. Laccase (1.675 nkat/mg of MBAX) was used as a cross-linking agent. The
121 gels were allowed to develop for 6 h at 25 °C.

122 2.3. Rheological properties

123 The rheological tests were performed by small amplitude oscillatory shearing using a
124 strain-controlled rheometer (Discovery HR-2 rheometer, TA Instruments, New Castle, DE,
125 USA) as reported by Vansteenkiste et al. (2004). MBAX gelation was studied for 6 h at 25
126 °C. All measurements were carried out at a frequency of 0.25 Hz and 5% strain (linearity
127 range of viscoelastic behavior). The mechanical spectra of gels were obtained by
128 conducting a frequency sweep from 0.01 to 100 Hz at 25 °C.

129

130

131 2.4. Phenolic Acids Content

The FA, di-FA and tri-FA contents in the MBAX gels were quantified by RP-HPLC after a de-esterification step as described by Vansteenkiste et al. (2004). An Alltima (Alltech, Deerfield, IL, USA) C18 column (250 × 4.6 mm) and a photodiode array detector Waters 996 (Millipore Co., Milford, MA, USA) were used. Detection was followed by UV absorbance at 320 nm.

2.5. Swelling and structural parameters

The MBAX-4 and MBAX-6 were allowed to swell as described by Carvajal-Millan, Landillon, et al. (2005). The equilibrium swelling was reached when the weight of the samples changed by no more than 3% (0.06 g). The swelling ratio (q) was calculated as follows:

$$q = (W_s - W_{MBAX})/W_{MBAX},$$

where W_s is the weight of swollen gels and W_{MBAX} is the weight of the MBAX-4 or MBAX-6 (w/v) gels. From the swelling measurements, the molecular weight between two cross-links (M_c), the cross-linking density (ρ_c) and the mesh size (ξ) values of the MBAX-4 and MBAX-6 gels were calculated as reported by Carvajal-Millan, Guilbert, Morel, & Micard, 2005, using the model of Flory & Rehner (1943) modified by Peppas & Merrill (1976) for gels in which the cross-links are introduced in solution.

2.6. Scanning electron microscopy

The microstructure of the MBAX-4 and MBAX-6 gels and surface morphology of the residual MBAX gels after 18 and 36 h fermentation by a mixture of *Bifidobacterium longum* and *Bifidobacterium adolescentis* were studied by scanning electron microscopy

153 (SEM) (JEOL JSM-7401F, Peabody, MA, USA) without coating at low voltage (10 kV).

154 SEM images were obtained in secondary and backscattered electron image modes.

155 2.7. Degradation of MBAX gels

156 2.7.1. Organism and culture conditions

157 The bacterial strains used (*Bifidobacterium adolescentis* ATCC 15703 and *Bifidobacterium*
158 *longum* ATCC 15708) were purchased from American Type Culture Collection (Manassas,
159 VA, USA). The bacteria were maintained at -80 °C in glycerol stock solutions. The
160 bacterial suspensions were thawed, and the bacteria were revived by culturing in the basal
161 media as described previously by Hughes et al. (2007). The strains were incubated in the
162 broth under anaerobic conditions at 37 °C until an optical density of one (OD= 1) was
163 reached. Afterward, they were used as an inoculum for growth studies on MBAX gels.

164 2.7.2. In vitro fermentation

165 For the fermentation analysis, the MBAX-4 and MBAX-6 gels were formed in Hungate
166 tubes and autoclaved at 121 °C for 15 min. MBAX without gelation was used as a positive
167 control. The MBAX and MBAX gels were mixed with autoclaved basal media to give a
168 final concentration of 5 g/L (w/v) MBAX as a sole carbon source. 100 µL Oxyrase
169 (Oxyrase Inc. Mansfield, OH, USA) was added as the broth to remove oxygen from the
170 microenvironment of the test tube. The tubes were sealed anaerobically by flushing the
171 headspace with carbon dioxide and placed at 4 °C overnight. These Hungate tubes were
172 inoculated with a 1% (v/v) mixture of two bifidobacteria species (*B. adolescentis* and *B.*
173 *longum*; ratio 1:1) and incubated in the dark without shaking at 37 °C for 18, 36 and 72 h.
174 Fermentations were carried out in triplicate, and duplicate blank tubes containing no

substrate were included for each time point. The OD (650 nm) was measured at 18, 36 and 72 h after inoculation. The pH was measured at the end of each different growth period, and random samples were examined microscopically to exclude contamination by the bacterial cultures.

2.7.3 Fermentation product analysis

Before analysis, at time 0 of fermentation, one milliliter of each MBAX gel was treated with 0.5 mL 0.5 N NaOH for 15 min at 25°C to disrupt the gels by releasing the phenolic bridges. The pH was then immediately adjusted to 7 to prevent MBAX chain degradation. The samples for the other fermentation time points were only centrifuged (15,000 g, 4 °C, 5 min) to remove the bacteria. The changes in the molecular weight distribution of the MBAX and MBAX gels at different times of fermentation (0, 18, 36, 72 h) were determined by SE-HPLC at 38 °C using a TSKGel G5000PWXL column (7.8 x 300 mm) in combination with a PWX-guard column (6 x 40 mm) (Tosoh Bioscience, Stuttgart, Germany). Molecular weights were estimated after universal calibration with pullulans as standards (P50 to P800). Isocratic elution was conducted at 0.6 mL/min with 0.01 M LiNO₃ filtered through 0.2 µm. Differential refractometry was used for detection.

Residual carbohydrates in the freeze-dried fermentation residues and monosaccharides in the supernatant of the centrifuged samples were quantified by RP-HPLC as described by Carvajal-Millan et al. (2007). The main monosaccharides of MBAX (arabinose, xylose, galactose and glucose) were used as standards.

Short-chain fatty acids (SCFA) were analyzed based on Pastell, Westermann, Meyer, Tuomainen, & Tenkanen (2009). The SCFA analysis was carried out using GC (Clarus 580, PerkinElmer, Waltham, MA, USA) with a flame ionization detector and a capillarity

column (Elite-FFAP 30 m x 0.50 mm I.D; film thickness, 1 μ m). The SCFA in the samples was quantified using external calibration curves of acetic, propionic and butyric acids, and the results were expressed in μ g/mL.

2.8. Statistical analysis

The results are expressed as the means $\pm$ Standard Deviation (S.D.) from three replicates. The significance of differences was determined by analysis of variance (ANOVA) (OriginPro software, version 8.6. Originlab Corporation, USA) followed by Tukey's test and the results were considered to be statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Features of MBAX gels

3.1.1 Rheological properties and covalent cross-links

The formation of gel networks was monitored by storage (G') and loss (G'') modulus changes in 4 and 6% (w/v) AX solutions undergoing oxidative gelation by laccase (1.675 nkat/mg of MBAX) (Fig. 1a). The gelation profile vs. time exhibited an initial increase of the G' modulus, followed by a plateau region resulting from the sol-gel transition. By increasing the MBAX concentration, the gelation time (time $G' = G''$, i.e., $\tan \delta = 1$) decreased and the gelation rate (rate of G' development) increased. This relationship could be associated with a higher density of polysaccharide chains enhancing the probability of contact between ferulic acid free radicals, which is an essential requirement for the establishment of a three-dimensional AX network (Carvajal-Millan, Guilbert, Morel, & Micard, 2005; Rattan, Izydorczyk, & Biliaderis, 1994). The G' values (215 Pa) of the

MBAX gel at 4% (w/v) were higher than those reported for laccase-induced maize bran gel
 (20 Pa) or waste water arabinoxylans gel (2 Pa) at the same concentration, though MBAX-4
 had a similar initial FA content to both of these gels (0.23 and 0.37 $\mu\text{g}/\text{mg}$ AX,
 respectively) (Hernández-Espinoza et al., 2012; Niño-Medina et al., 2009). Such behaviors
 might have their origin in the structural and conformational characteristics of AX
 molecules. The increase in the MBAX concentration from 4 to 6% (w/v) in the gel resulted
 in an approximately two-fold increase in the value of G' (Fig. 1a). These results were in
 agreement with data reported previously by Rattan et al. (1994), who found linear
 relationships between G' (plateau values) and polymer concentration in samples of wheat
 AX. This G' vs. polymer concentration trend has also been observed in chemically cross-
 linked alginate gels (Choudhary & Bhatia, 2012) and dextran-lactate gels cross-linked by
 stereocomplex formation (De Jong et al., 2001). The mechanical strength of the matrix is
 known to have a direct bearing on drug delivery characteristics (Coviello et al., 2005;
 Woolfson, Malcolm, Campbell, Jones, & Russell, 2000). The mechanical spectra of the
 MBAX-4 and MBAX-6 gels after 6 h of gelation are shown in Fig. 1b. Though the MBAX-
 6 gel exhibited a solid-like behavior with $G' > G''$ and a plateau towards the low-frequency
 range (0.01 to 10 Hz), G' decreased steeply with increasing frequency (beyond 40 Hz). This
 behavior is typical of strong gels that present rupture and fail at large deformations. The
 MBAX-4 gel showed a linear G' independent of the frequency of oscillation and a G'' that
 was drastically lower than G' and linearly dependent on the frequency (Ross-Murphy &
 Shatwell, 1993). The dependence of G'' on the frequency has been related to the
 participation of physical entanglement interactions in the maturation of the gel and the
 various levels of connectivity and junction zones of disparate lengths and strengths within
 the three-dimensional network (Kasapis, 2008; Picout & Ross-Murphy, 2003). Hence, these

244 differences can be attributed to the covalent cross-linking distribution and the physical
245 entanglement of AX chains in MBAX gels as a function of AX concentration. Similar
246 mechanical spectra have been previously reported for strong gels with potential uses as
247 carriers of proteins and drugs systems (Choh, Cross, & Wang, 2011; Choudhary & Bhatia,
248 2012; De Jong et al., 2001).

249 **Fig. 1.** (a) Monitoring the storage (G') and loss (G'') modulus of MBAX solution at 4 and
250 6% (w/v) during gelation by laccase (1.675 nKat/mg of MBAX) at 0.25 Hz. (b) Mechanical
251 spectrum of MBAX gels after 6 h gelation. Rheological measurements were carried out at
252 25 °C and 5% strain.

254 The extent of covalent cross-linking in the MBAX gels was determined by the content of
255 total di-FA and tri-FA after 6 h of gelation (Table 1). The amounts of di-FA and tri-FA
256 produced during gelation never compensated for the loss of FA monomer. Indeed, at the
257 end of gelation, 80 and 70% of the FA initially present in MBAX was oxidized; by

contrast, only 32 and 34% was recovered as a combination of di-FA and tri-FA in MBAX-6 and MBAX-4, respectively. Nevertheless, the $\tan \delta$ (G''/G') values confirmed the formation of an elastic covalent system for both gels (Fig. 1a). These behaviors in MBAX gels have been previously reported by several authors (Carvajal-Millan et al., 2007; Lapierre, Pollet, Ralet, & Saulnier, 2001; Niño-Medina et al., 2009). These authors attributed such phenomena to the formation of higher ferulate cross-linking structures unreleased by mild alkaline hydrolysis and/or to the participation of lignin residues coupled with FA monomers in the formation of MBAX gels. At the end of gelation, the 8-5' form (especially benzofuran) contributed approximately 68 - 79% of the total di-FA in MBAX-4 and MBAX-6, whereas the 5-5' and 8-O-4' forms contributed 22 - 12% and 10 - 9%, respectively. The predominance of 8-5' di-FA was also observed in wheat flour AX treated with a peroxidase/H₂O₂ system or laccase (Carvajal-Millan, Landillon, et al., 2005; Figueroa-Espinosa et al., 1999). Nevertheless, the 5-5' di-FA structure has been reported to be predominant in AX gels from maize bran (Carvajal-Millan et al., 2007; Niño-Medina et al., 2009). In a previous study, Hatfield & Ralph (1999) reported that only 5-5' di-FA can be formed intramolecularly in arabinoxylans, i.e., on the same polysaccharide chains. In this study, the greater proportion of the 5-5' di-FA formed in the MBAX-4 gel (22%) compared with the MBAX-6 gel (12%) might have indicated that in MBAX-4, the intrachain bonds were favored and thus the polymer network showed decreasing continuity. These results may partly explain some differences in the mechanical properties of the gels.

Table 1. Swelling and structural parameters of MBAX gels.

	q^a (g H ₂ O/g MBAX)	$M_c^b \times 10^{-3}$ (g/mol)	$\rho_c^c \times 10^{-6}$ (mol/cm ³)	ξ^d (nm)	FA (μg/mg MBAX)	di-FA
MBAX-4	18.11 ± 1.23	36 ± 0.60	39 ± 0.05	179 ± 5.23	0.054 ± 0.017	0.031 ± 0.011
MBAX-6	9.36 ± 1.86	26 ± 0.31	54 ± 0.06	110 ± 0.32	0.074 ± 0.002	0.044 ± 0.001

^a Swelling ratio; ^b Molecular weight between two cross-links; ^c Cross-linking density;

^d Mesh size.

3.1.2 Swelling and structure of gels

The swelling ratio (q) of gels is an important parameter that describes the amount of water stored within the gel network. The swelling behavior of the MBAX gels at 4 and 6% (w/v) was assessed in water for 15 h at 25 °C, with the equilibrium being reached between 8 and 6 h in MBAX-4 and MBAX-6, respectively. The q value (in g water/g of MBAX dry weight) in the MBAX gels decreased from 18 to 9 when the MBAX concentration in the gel increased from 4 to 6% (w/v) (Table 1). The q value found for the MBAX-4 gel was similar to the value reported for maize bran AX gels (3.5% (w/v), $q = 20$) (Berlanga-Reyes, Carvajal-Millan, Caire Juvera, et al., 2009). The lower swelling ratio value registered at the highest MBAX concentration in the gel (MBAX-6) may have been related to the more compact polymeric structure that limited the water absorption (Meyvis, De Smedt, Demeester, & Hennink, 2000; Ross-Murphy & Shatwell, 1993). A similar effect of polysaccharide concentrations on the swelling capacity of a gel has been reported for water-extractable AX (0.5 to 2 % (w/v), q values ranging from 231 to 69) or sugar beet pectin (2

300 to 5% (w/v), q values ranging from 120 to 35) after treatment with laccase (Carvajal-
301 Millan, Guilbert, et al., 2005; Micard & Thibault, 1999).

302 The cross-sectional microstructures of the MBAX-4 and MBAX-6 gels before swelling
303 were observed by scanning electron microscopy (SEM). Cross-sectional SEM images of the
304 MBAX gels are shown in Fig. 2. Both gels showed a three-dimensional and heterogeneous
305 network structure formed by the aggregation of nodular structures into clusters. MBAX-6
306 (Fig. 2b) presented a more compact microstructure in comparison with the geometries and
307 irregular pores sizes of the MBAX-4 structure (Fig. 2a). These results are consistent with
308 the structural parameters calculated from the equilibrium swelling test. The molecular
309 weight between two cross-links (M_c), the cross-linking density (ρ_c) and the mesh size (ξ)
310 values for MBAX-6 and MBAX-4 are presented in Table 1. When the MBAX
311 concentration increased in the gel from 4 to 6%, M_c and ξ decreased, leading to an increase
312 in the cross-linking density ρ_c . Similar M_c , ρ_c and ξ values have been reported in 3 to 5 %
313 AX gels (Berlanga-Reyes, Carvajal-Millan, Caire Juvera, et al., 2009; Carvajal-Millan,
314 Guilbert, et al., 2005). The structural parameters are important in determining the physical
315 properties of the gels, including their mechanical strength, the diffusivity of the released
316 molecule, and their degradability (Hoffman, 2012). In hydrogels for drugs and protein
317 delivery or cell encapsulation, a typical ξ range of 5 to 300 nm has been reported for the
318 swollen state (Carvajal-Millan et al., 2006; Choh et al., 2011; Choudhary & Bhatia, 2012).
319 The ξ values found for MBAX-4 and MBAX-6 are within this range. The results discussed
320 above indicate that by modifying the concentration of polysaccharides in the network, gels
321 with different rheological and structural properties can be obtained.

Fig. 2. SEM image showing the microstructures of cross-sectional MBAX-4 (a) and MBAX-6 (b) networks at 350x magnification.

3.2 Degradation of MBAX gels

The surface structures of degraded MBAX-4 and MBAX-6 hydrogels were studied by SEM and are presented in Fig. 3. At least two independent replicates of each sample were analyzed for their surface morphologies, and similar results were obtained for the replicates. Fig. 3a-b show the surface structures of MBAX-4 and MBA-6, respectively, after 18 h of incubation under the same conditions as those used for fermentation but without the bacterial strains. The changes in the surface morphology of the MBAX-4 and MBAX-6 hydrogels were observed as a function of their exposure time to *Bifidobacterium* culture (a mixture of *Bifidobacterium longum* and *Bifidobacterium adolescentis*). SEM images taken after 18 h and 36 h of incubation with the *Bifidobacterium* culture showed the extent of the gel morphological changes (Fig. 3c-f). For both samples, the undegraded MBAX gels presented relatively smooth and continuous surface structures without holes. The degraded gels lost this structure and exhibited multiple cavities in their microstructure, which increased in size and number with the time of incubation due to the enzymatic activity of *B. longum* and *B. adolescentis*. However, at 18 h of incubation, differences in the size of the cavities were observed between the MBAX gels (Fig. 3d vs. Fig. 3c). At 0 h of

incubation, MBAX-6 has more cross-linked networks and a more compact structure compared to MBAX-4 (Table 1 and Fig. 2), which may have reduced the accessibility of the gel to enzymes and thus slowed down its degradation. Nonetheless, at 36 h of incubation, the structure of both MBAX gels appeared to be collapsed rather than porous, as shown in Fig. 3e-f. Therefore, it is probable that after an initial surface erosion, the enzymes diffused into the polymeric network and thus caused the degradation of the most internal sites. Similar results were obtained for chemically crosslinked dextran gels that were degraded by microbial enzymes (Brøndsted et al., 1998).

349

Fig 3. Scanning electron micrographs showing the surface morphologies of covalently cross-linked MBAX-4 (a,c,e) and MBAX-6 (b,d,f) gels: (a-b) without degradation and after; (c-d) 18 and (e-f) 36 hours of incubation with a mixture of *Bifidobacterium* strains (*B. longum*, *B. adolescentis*). All samples are at 350x magnification.

354 The degradation of MBAX gels with different structures was assessed by measuring the
355 residual AX molecular weights (Mw) and free monosaccharides in the fermentation media.
356 During the degradation experiments, MBAX chains with different Mw (Fig. 4) and a high
357 amount of free monosaccharides were released (data not shown). The changes in the
358 residual AX Mw in ungelified MBAX and MBAX gels at different fermentation times were
359 registered by gel permeation chromatography (Fig. 4). The AX elution profile at time 0
360 represents the Mw distribution of ungelified MBAX and MBAX gels without bacterial
361 chain degradation. The Mw loss of MBAX gels was observed only in the presence of both
362 strains of *Bifidobacterium*. These results indicated that the presence of both bacteria is
363 necessary to break down the gel structure. Previous research has suggested a possible cross-
364 synergy between *B. longum* and *B. adolescentis* in the degradation of low molecular weight
365 arabinoxylans (Pastell et al., 2009; Wang, Sun, Cao, & Wang, 2010). The ungelified
366 MBAX showed over 80% of molecular weight loss after 18 h incubation and Mw
367 distributions of less than 50 kDa. This pattern was similar for different times of incubation
368 of ungelified MBAX (Fig 4a). Fig. 4c shows that MBAX-6 was slowly degraded and had a
369 Mw distribution of less than 50 kDa after 72 h of incubation, whereas MBAX-4 presented
370 this pattern after 36 h of incubation (Fig. 4b). However, in the first 18 h of incubation, a
371 fraction of both gels had Mw greater than 110 kDa. Hopkins et al. (2003) reported that the
372 breakdown of the xylan backbone in crosslinked AX was more resistant to degradation
373 compared with the uncrosslinked AX due to the restricted access of xylanolytic enzymes.
374 Thus, the degradation of the gel in the early hours of incubation was restricted by the ability
375 of the enzymes to access their target sites.

376

377 **Fig. 4.** HPSEC elution profiles of hydrolysis products of (a) ungelified MBAX, (b) MBAX-
378 4 gel and (c) MBAX-6 gel degradation by a mixture of *Bifidobacterium* strains (*B. longum*
379 and *B. adolescentis*) after incubation for 0 (t_0), 18 (t_1), 36 (t_2) and 72 (t_3) h. The elution
380 profiles of pullulan molecular weight markers (kDa) were used for calibration and are
381 shown at the top.

19

382 Changes in the degree of substitution were also observed during the fermentation, as shown
383 in Fig. 5a. The A/X ratio decreased with time in MBAX solution, MBAX-4, and MBAX-6
384 but increased after 36 h for the MBAX solution only. The differences observed after 36 h
385 between the MBAX solution and the MBAX gels could be associated with a more rapid
386 initial removal of the arabinose side chains from the ungelified MBAX compared to the
387 MBAX gels. Once the branched regions of MBAX were removed, the xylan backbone was
388 hydrolyzed by bacterial xylanases, as reflected the increase in the A/X ratio. Hence, the
389 MBAX solution, MBAX-4, and MBAX-6 were degraded by a debranching mechanism
390 (Rose et al., 2010). The MBAX-4 and MBAX-6 gels showed different slopes for the linear
391 decrease of the A/X ratio, in which the A/X ratio decreased much more rapidly in the
392 MBAX-4 gel than in the MBAX-6 gel. This difference was mainly related to the low
393 average mesh size (Table 1), compact microstructure (Fig. 2) and probably higher content
394 of intrachain di-FA in MBAX-6 compared to MBAX-4. Rosa et al. (2013) observed an
395 effect of the pore size on the in vitro fermentation of wheat aleurone with a high content of
396 insoluble AX. Moreover, Park et al. (2014) reported that the main factor causing the high
397 resistance to degradation of hyaluronic acid gels is the low swelling ratio resulting from a
398 high concentration of polysaccharides and a high degree of crosslinking. Hence, the slow
399 removal of the arabinose side chains from MBAX-6 may have been due to its more
400 compact structure, which slowed down enzymes penetration.

Fig. 5. Changes in Arabinose/Xylose ratio during *in vitro* fermentation of ungelified MBAX and MBAX-4 and MBAX-6 gels by a mixture of *Bifidobacterium* strains (*B. longum*, *B. adolescentis*).

The results above are consistent with the production of short-chain fatty acids registered during the *in vitro* fermentation by the *Bifidobacterium* mix (Fig. 6). All three samples produced significantly higher concentrations of total SCFA in comparison to the control cultures at different times (MBAX-free substrate). The SCFA production increased with the time that the MBAX-4 and MBAX-6 gels were fermented. In contrast, the SCFA production in the ungelified MBAX remained constant from 36 to 72 h. Ungelified MBAX produced a significantly ($p < 0.05$) higher amount of SCFA in comparison to the MBAX gels at all fermentation times. At 18 h, both MBAX gels showed no significant differences in the amount of SCFA produced. However, at 36 and 72 h, MBAX-6 presented a lower amount of SCFA than MBAX-4. The total SCFA decreased significantly when the degree of cross-linking increased in the MBAX gels. Acetic acid was the main acid produced,

417 followed by propionic and butyric acids. The ungelified MBAX and MBAX-4 produced
 418 more acetic and propionic acids than MBAX-6, while butyric acid production was not
 419 significantly different among the three fermented samples. The SCFA profiles resulting
 420 from the fermentation of the ungelified MBAX and MBAX gels were similar to the one
 421 reported for alkali-soluble arabinoxylans from maize (Rose et al., 2010). These results
 422 indicate that a certain portion of the AX chains released during the degradation of MBAX
 423 gels was fermentable and that carbohydrates were efficiently converted to SCFA by the
 424 *Bifidobacterium* mix.

425
 426 **Fig. 6.** Total short-chain fatty acid (SCFA) production during fermentation of ungelified
 427 MBAX, MBAX-4 gel and MBAX-6 gel by a mixture of *Bifidobacterium* strains (*B.*
 428 *longum*, *B. adolescentis*) after incubation for 18, 36 and 72 h. Different letters (a to e)
 429 indicate significant differences between samples ($p < 0.05$).

430

431 4. Conclusions

432 Covalently cross-linked arabinoxylan gels with different structures were obtained by
433 increasing the concentration of AX undergoing oxidative gelation by laccase. The presence
434 of a more compact structure with a higher crosslinking density allowed for a slower
435 degradation of the three-dimensional gel structure by fermentation with a mixture of
436 *Bifidobacterium*. The ability to modulate the structural parameters of the AX gels to change
437 their biodegradability suggest that covalently cross-linked arabinoxylan gels could be used
438 for the design of controlled delivery systems activated by the colon microbiota. The *in vitro*
439 and *in vivo* simulation of arabinoxylan gels degradation in the colon will be further
440 investigated.

441

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448

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