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# Fermentation of Ferulated Arabinoxylan Recovered from the Maize Bioethanol Industry

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**Abstract:** Maize by-product from the bioethanol industry (distiller's dried grains with solubles, DDGS) is a source of ferulated arabinoxylan (AX), which is a health-promoting polysaccharide. In the present study, AX from DDGS was fermented by a representative colonic bacterial mixture (*Bifidobacterium longum*, *Bifidobacterium adolescentis*, and *Bacteroides ovatus*), and the effect of the fermented AX (AX-f) on the proliferation of the cell line Caco-2 was investigated. AX was efficiently metabolized by these bacteria, as evidenced by a decrease in the polysaccharide molecular weight from 209 kDa to < 50 kDa in AX-f, the release of ferulic acid (FA) from polysaccharide chains (1.14 µg/mg AX-f), and the short-chain fatty acids (SCFA) production (277 µmol/50 mg AX). AX-f inhibited the proliferation of Caco-2 cells by 80–40% using concentrations from 125–1000 µg/mL. This dose-dependent inverse effect was attributed to the increased viscosity of the media due to the polysaccharide concentration. The results suggest that the AX-f dose range and the SCFA and free FA production are key determinants of antiproliferative activity. Using the same polysaccharide concentrations, non-fermented AX only inhibited the Caco-2 cells proliferation by 8%. These findings highlight the potential of AX recovered from the maize bioethanol industry as an antiproliferative agent once fermented by colonic bacteria.

**Keywords:** ferulated arabinoxylan; fermentation; ferulic acid; colon cancer; antiproliferative agent



**Citation:** Mendez-Encinas, M.A.; Valencia-Rivera, D.E.; Carvajal-Millan, E.; Astiazaran-Garcia, H.; Micard, V.; Rascón-Chu, A. Fermentation of Ferulated Arabinoxylan Recovered from the Maize Bioethanol Industry. *Processes* **2021**, *9*, 165. https://doi.org/10.3390/pr9010165

Received: 11 November 2020
Accepted: 12 January 2021
Published: 18 January 2021



## 1. Introduction

The bioethanol industry has been augmenting worldwide, especially in the United States, with maize being the primary raw material used for this purpose in that country. Distiller's dried grains with solubles (DDGS), constituted from the non-starch maize components such as maize bran, is generated in huge magnitude as a by-product during bioethanol production. DDGS is sold for a very low price and is mainly used in animal production as a nutrient supplement. Therefore, the development of value-added products from DDGS has been of interest in recent years. One of the principal components of DDGS is a dietary fiber and more precisely a ferulated arabinoxylan (AX). AX is the main non-starch polysaccharide from cereal grains with a basic structure consisting of a linear β-(1-4)-linked D-xylopyranosyl backbone chain with some α-L-arabinofuranose units attached to the O-2 and O-3 positions of xylose units [1]. In addition, some ferulic acid (FA) residues can be esterified to the arabinose side chains through O-5 [2] (Figure 1). AX reaches the colonic region where it is fermented by the gut microbiota, resulting in the production of short-chain fatty acids (SCFA) as end products [3]. SCFA are well-known to exert positive physiological effects on host health. In particular, butyric acid plays a role in

the prevention of colon cancer, as it is the primary substrate for colonocytes and promotes the health of the gut barrier [4]. Prior studies have documented the antiproliferative effect of SCFA, butyrate, and acetate on colorectal cancer cells through different mechanisms, such as gene expression inhibition and apoptosis [5,6]. Moreover, Glei et al. [7] reported the antiproliferative effect of wheat AX fermentation supernatants on the human colon cancer cell line HT-29. The protective effect of phenolic acids, to which FA belongs, on colon carcinogenesis has also been reported by several authors [8–11]. FA has been demonstrated to inhibit the proliferation of the human colon cancer cell line Caco-2 by affecting the cell cycle, explicitly inducing a delay in the S phase and dysregulating genes implied in the cell cycle control [9,10]. AX is mainly degraded by *Bacteroides* and *Bifidobacterium*, as they produce the enzymes required for AX degradation [12,13]. A symbiotic effect between *Bifidobacterium longum* and *Bifidobacterium adolescentis* in arabinoxylo-oligosaccharides (AXOS) degradation has been suggested [14]. Previous studies have documented that cross-linked AX fermentation promotes the growth of probiotics such as *Lactobacillus* and *Bifidobacterium* [13,14], while the development of particular strains such as *Bacteroides* is not favored [12]. Thus, AX fermentation could exert positive effects on host health by producing beneficial metabolites and modulating the microbiota composition.

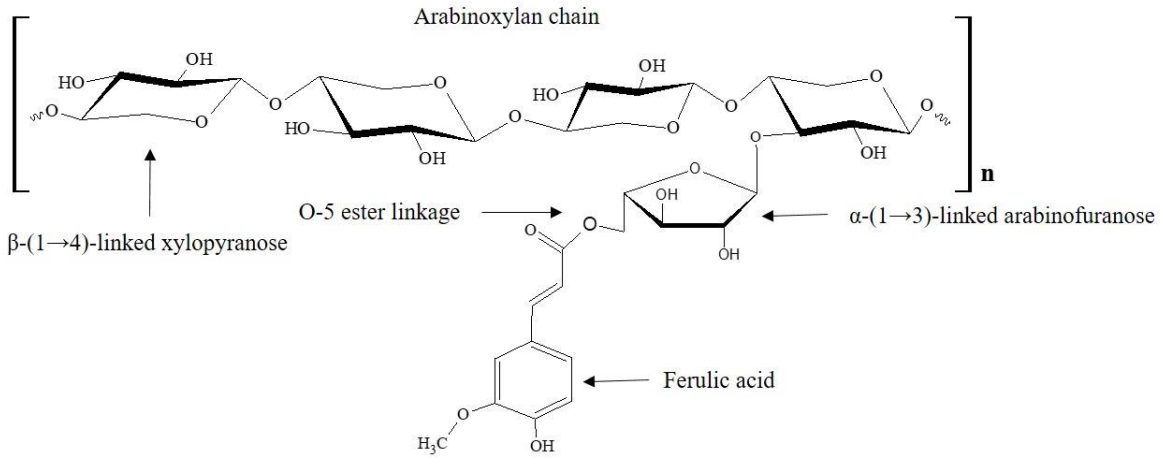


**Figure 1.** Chemical structure of maize ferulated arabinoxylan.

AX fermentation may be affected by the polysaccharide's structural characteristics, which give rise to physiological functions and exert diverse impacts on the microbiota [3]. Several features, such as the molecular weight, the degree of polymerization, the arabinose substitution, and the ferulation degree, influence the fermentability of AX and impact on SCFA production [3]. The presence of FA esterified to AXOS hinders enzyme activity and inhibits their degradation and subsequent fermentation [15]. Moreover, to our knowledge, no report exists about the effect of the fermentation products of AX recovered from DDGS on the proliferation of human colon cancer cells Caco-2. Therefore, this study aimed first to investigate the in vitro fermentation of AX from DDGS by a representative colonic bacterial mixture (*B. longum*, *B. adolescentis*, and *Bacteroides ovatus*), and second, to evaluate for the first time the effect of this fermented AX on the proliferation of the human colon cancer cell line Caco-2.

## 2. Materials and Methods

### *2.1. Materials*

AX was recovered from DDGS and characterized according to previous report [16]. The AX presented an arabinose-to-xylose ratio (A/X) of 1.1, a protein content of 8.2%, and an average molecular weight of 209 kDa. The polysaccharide contained 64% pure AX (sum A + X) on a dry basis (d.b.). Laccase (E.C.1.10.3.2) from *Trametes versicolor* and all the chemical products used were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA).

### 2.2. Organisms and Culture Conditions

The bacterial strains used were *B. longum* (ATCC® 15708TM), *B. adolescentis* (ATCC® 15703TM), and *B. ovatus* (ATCC® 8483TM), purchased from the American Type Culture Collection (Mannasas, VA, USA). The bacteria were preserved in 10% glycerol stock solutions at −80 °C. The propagation of bacteria was performed according to van Laere et al. [17] and Crittenden et al. [18] with some modifications. For bacterial propagation, bifidobacteria were cultivated once in fresh MRS (deMan, Rogose and Sharpe) medium and twice in BSM (Bifidus Selective Medium) medium, and *B. ovatus* was cultivated three times in TSB (Tryptic Soy Broth) medium by inoculating 1 mL of the bacterial solution into the next solution. Afterward, the bacteria were grown in basal medium (culture medium with no added carbon source), as described by Hughes et al. [13]. The bacteria were first inoculated (0.5 mL) into glass tubes with 5 mL of basal medium containing 1% (*w*/*v*) arabinose/xylose (A/X, 1:1) as the sole carbon source and incubated under anaerobic conditions at 37 °C for 24 h and then inoculated (0.5 mL) in 5 mL of 1% (*w*/*v*) AX in basal medium. The overnight full-grown cultures were used as inocula for the AX fermentation experiment.

### 2.3. In Vitro Fermentation of AX

The in vitro fermentation experiments were performed following the method reported by Martínez-López et al. [19], with some modifications. AX was autoclaved at 121 °C for 15 min and then mixed with sterile basal medium to obtain a final concentration of 5 g/L (*w*/*v*) AX as the sole carbon source. The dissolved oxygen in the culture media was removed by adding Oxyrase (Oxyrase Inc., Mansfield, OH, USA) to the samples. The media were inoculated with 4.5% (*v*/*v*) of a bacterial mixture (*B. longum*, *B. adolescentis*, and *B. ovatus*, ratio 1:1:1) and incubated under anaerobic conditions for 48 h at 37 °C. The fermentations were performed in triplicate, and basal medium with no added carbon source was used as Control. The bacterial growth was measured by monitoring the optical density (OD) of samples immediately after inoculation and after 18, 24, 42, and 48 h of fermentation. Aliquots of 200 µL of culture were placed in 96-well microplates, and the OD was registered at 600 nm via a microplate reader (Thermo Scientific MultiSkan Go, Madrid, Spain). Additionally, the pH of the culture medium was monitored during the experiment, and the strains were checked microscopically to control morphology conformation before and after fermentation. At the end of fermentation, the culture medium was centrifuged at 2000× *g* for 15 min at 4 °C. The fermented AX (AX-f) was recovered and stored at −80 °C for further analyses.

### 2.4. Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR spectra of AX and lyophilized AX-f were obtained in absorbance mode (400 and 4000 $cm^{-1}$) at 4 $cm^{-1}$ resolution using a Nicolet iS50 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

### 2.5. Molecular Weight Distribution

The molecular weight distribution of AX and lyophilized AX-f were determined by size exclusion-high-performance liquid chromatography (SE-HPLC) at 38 °C using a TSK gel (Polymer Laboratories, Shropshire, United Kingdom) G5000 PWXL column (7.8 mm × 300 mm). Isocratic elution was performed at 0.6 mL/min with 0.1 M $LiNO_3$ (filtered at 0.22 µm). Twenty µL of each sample (0.1% *w/v* in 0.1 M $LiNO_3$ filtered through a 0.22 µm pore size filter) was injected and detection was performed using a Waters 2414 refractive index detector [19]. The molecular weights were estimated using a pullulan standard (P50 to P800) calibration curve. The elution profile of the basal media was also analyzed to identify and compare the observed peaks.

### 2.6. Short-Chain Fatty Acids (SCFA)

SCFA analysis was determined following a previous protocol [20] with modifications. The pH of the samples (Control and AX-f) was adjusted to 2 with 5 M HCl and maintained

at 25 °C for 10 min with occasional shaking. 4-Methyl valeric acid (12% *v/v* in formic acid) was added to the samples (1 mM) as an internal standard and injected for analysis. The SCFA determination was performed in a GC (Clarus 580, PerkinElmer, Waltham, MA, USA) with a flame ionization detector and a capillarity column (Elite-FFAP 30 m × 0.50 mm I.D.; film thickness, 1 µm). The SCFA concentration was determined using calibration curves of acetic, propionic, and butyric acids. The results were expressed in mM.

### *2.7. Phenolic Acids*

FA, di-FA, and tri-FA were determined after a de-esterification step by Reversed-phase HPLC, as reported elsewhere [21,22] using an Alltima C18 column (250 mm × 4.6 mm; Alltech Associates, Inc., Deerfield, IL, USA). A photodiode array detector (Waters 996, Waters Co., Milford, MA, USA) was used. Detection was followed by UV absorbance (320 nm). For free FA, di-FA, and tri-FA determination, the same procedure was followed without a de-esterification step.

### *2.8. Antiproliferative Activity Assay*

#### 2.8.1. Cell Lines

The cancerous cell line Caco-2 [Caco2] (ATCC® HTB-37) (human colon cancer) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM (Dulbecco's modified Eagle's medium), containing 10% of fetal bovine serum, 100 U/mL of penicillin, 100 mg/mL streptomycin, and 1% of non-essential amino acids. Cells were preserved at 37 °C and 5% $CO_2$ in a humidified incubator (Thermo Fischer Scientific, San Jose, CA, USA) [23].

#### 2.8.2. Determination of Cell Proliferation

Before cell proliferation experiments, AX-f was obtained by centrifugation of samples at 4200× *g* for 15 min at 4 °C. Then, AX-f was centrifuged again at 16,000× *g* for 15 min at 4 °C. Afterward, AX-f was sterilized by filtration (0.22 µm) and stored at −80 °C for further analysis. The effect of the AX-f on the proliferation of the human colon cancer cell line Caco-2 was determined following the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [24] with some modifications [25]. Cells ($1 \times 10^4$ cells/50 µL) were seeded in 96-well microplates and incubated for 24 h at 37 °C in an atmosphere of 5% $CO_2$ to allow cell adhesion. After incubation, aliquots of AX-f dilutions in DMEM (50 µL) were added to the cells to obtain final concentrations from 125 to 1000 µg/mL, and were then incubated for 48 h at 37 °C. Basal medium with no added carbon source was used as a negative control, and the cytotoxic drug 5-fluorouracil (5-FU, 26 µg/mL) was used as a positive control in the antiproliferative assays. Non-fermented AX was also tested at concentrations previously reported in the normal human colon cell line CCD 841 CoN (125–1000 µg/mL) [26]. Within the last 4 h of incubation, 10 µL of MTT solution (5 mg/mL) was pipetted into each well. The cell viability was determined by the capacity of viable cells to reduce the tetrazolium salt to formazan crystals. The resulting purple precipitates formed were dissolved using acidic (0.4%) isopropyl alcohol. Samples absorbance was read at a test wavelength of 570 nm with a reference wavelength of 650 nm using an ELISA plate reader (Thermo Scientific MultiSkan Go, Madrid, Spain).

### *2.9. Statistical Analysis*

The results are presented as the means ± standard deviation (S.D) of three repetitions. The significance of differences was determined using an analysis of variance (ANOVA) with a Tukey–Kramer multiple comparison test ($p \leq 0.05$) (NCSS, 2007).

## 3. Results and Discussion

### *3.1. AX Fermentation*

Three strains, *Bifidobacterium longum*, *Bifidobacterium adolescentis*, and *Bacteroides ovatus*, were selected to perform the in vitro fermentation of AX, as they have been found to be ca-

pable of degrading this polysaccharide [3,12,19]. Bifidobacteria are well-known probiotics, and these two species (*B. longum* and *B. adolescentis*), in particular, have been reported to ferment AX [19]. In the present work, the ability of a mixture of *Bifidobacterium* and *Bacteroides* strains to ferment AX as a sole carbon source in the medium was investigated. The growth of bacteria on AX was assessed by measuring the optical density (OD) (Figure 2) and the pH of the culture during 48 h of incubation. The bacterial mixture was able to use AX as a sole carbon source, which was evidenced by an increase in the OD value, registering a maximum OD of ~0.5. AX fermentation was accompanied by an acidification of the culture medium, i.e., a pH decrease from 7 to 6 after 48 h of bacterial exposure, which was correlated with the increase in the OD value. The Control (no added carbon source) did not show any change in pH after the incubation period. Crittenden et al. [18] found that *B. longum* is more efficient than *Bacteroides* strains when using AX as a growth substrate. Additionally, evident growth of *Bifidobacterium breve* 286 on wheat AX was observed by Paesani et al. [27]. The degradation of AX by Bifidobacteria was demonstrated to be strain-dependent [28] and susceptible to cross-feeding between two strains, as previously reported with *B. longum* and *B. adolescentis* [14].

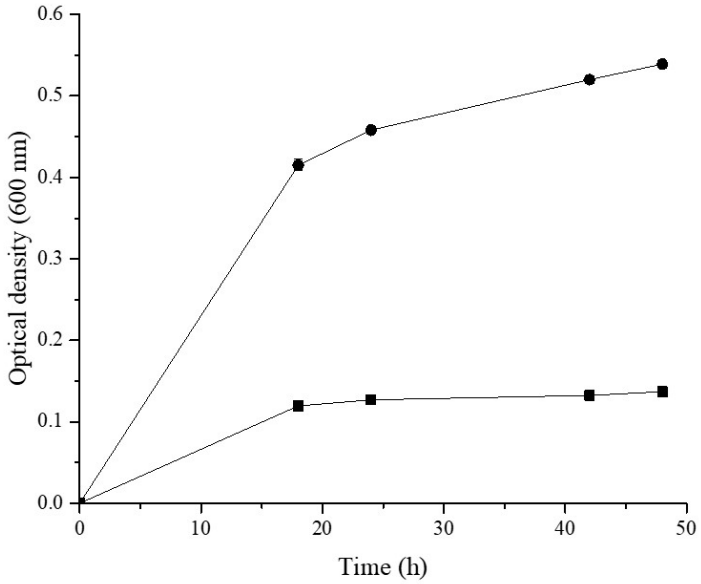


**Figure 2.** Growth of the bacterial mixture during the fermentation of (●) AX and (■) Control (no added carbon source).

In addition to OD and pH changes as indicators of fermentation, the molecular identity, the molecular weight distribution pattern, the esterified and non-esterified FA, di-FA, and tri-FA contents, and the SCFA production in AX and AX-f were determined.

### *3.2. FTIR Spectroscopy*

The FTIR spectra of AX, AX-f, and basal medium are presented in Figure 3. The maize AX spectrum (Figure 3a) registered a pattern with the typical bands previously reported for other AX [16,26]. In Figure 3a, the spectral pattern in the region 1200–900 $cm^{-1}$ revealed the presence of signals at 1045 and 898 $cm^{-1}$ attributed to the antisymmetric C-O-C stretching mode of the β-(1-4) linkage between the xylose units of the AX backbone [29,30]. In the absorbance region from 3500 to 1800 $cm^{-1}$, the bands observed at 3400 $cm^{-1}$ and 2900 $cm^{-1}$ are associated with OH stretching and $CH_2$ groups, respectively [31,32]. A similar FTIR band pattern was registered in AX-f (Figure 3b), indicating the presence of AX in AX-f. Thus, the degradation of AX by bacteria could have resulted in the production of AX chains with relatively low molecular weights (AX-f). The presence of additional peaks observed in the AX-f spectrum could be related to some basal medium components (Figure 3c) present

in the sample, especially the amide II band at 1548 $cm^{-1}$, which is mainly associated with polypeptide carbonyl stretching due to the peptone content in this medium.

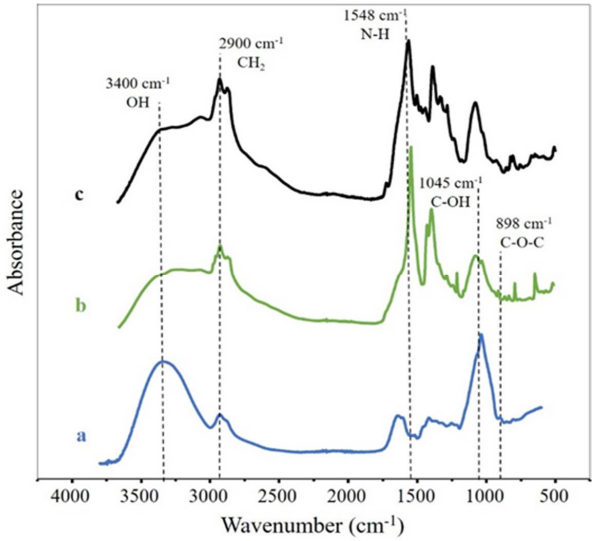


**Figure 3.** FTIR spectra of AX (**a**), AX-f (**b**), and basal medium (culture medium with no added carbon source) (**c**).

### *3.3. Molecular Weight Distribution*

The molecular weight (Mw) distribution pattern of AX, AX-f, and basal medium is presented in Figure 4. Maize AX presents apparent molecular weight in a broad range (polydispersion), with a major peak in the high-molecular-weight region (~209 kDa) and a minor peak in the low-molecular-weight region (<50 kDa). Similar SE-HPLC patterns have been previously reported for other AX [16]. The elution profile of AX-f registered only peaks with lower molecular weight fractions (<50 kDa) than those found in AX before fermentation, suggesting that the bacterial mixture was able to partially degrade the polysaccharide chain after 48 h of incubation.

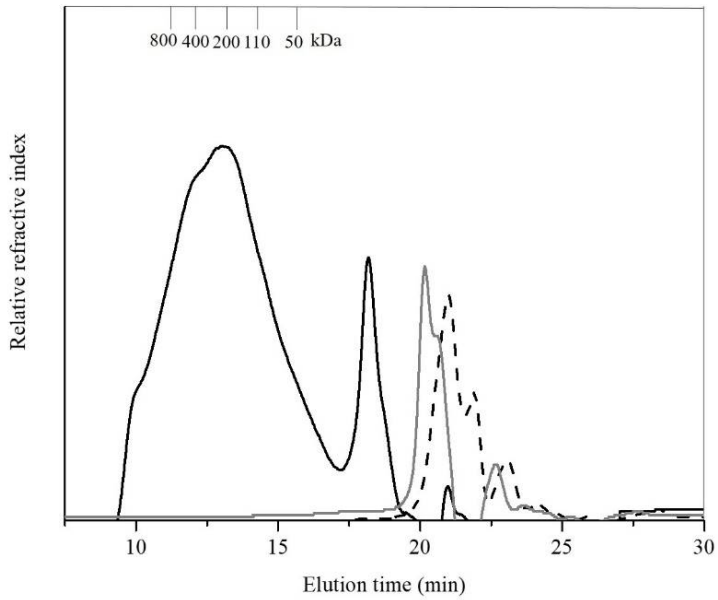


**Figure 4.** SE-HPLC elution profiles of AX (black line), AX-f (gray line), and basal medium (culture medium with no added carbon source, dotted line). Pullulan molecular weight markers (kDa) used as calibration scales are shown at the top.

### 3.4. Phenolic Acids Contents

The esterified and non-esterified FA, di-FA, and tri-FA contents in AX, AX-f, and the Control (no added carbon source) are shown in Table 1. AX-f contained 38% (2.07 µg/mg polysaccharide) of the esterified FA content registered in AX before fermentation (5.45 µg/mg polysaccharide), while only 9% of the di-FA quantified in AX was found in AX-f, and no tri-FA was detected in the latter. In addition, non-esterified FA was only detected in AX-f, indicating that bacterial feruloyl esterase was able to hydrolyze the ester linkage between the carboxylic group in FA and the C5-hydroxyl of the α-L-arabinose attached to the xylan backbone in AX [12].

**Table 1.** The esterified and non-esterified phenolic acids content in AX and AX-f.

| | | **Esterified** | |
|---|---|---|---|
| **Sample** | **FA** | **di-FA** | **tri-FA** |
| | | **µg/mg Sample (Dry Weight)** | |
| AX | 5.45 ± 0.09 | 0.35 ± 0.07 | 0.03 ± 0.00 |
| AX-f | 2.07 ± 0.03 | 0.03 ± 0.01 | nd |
| Control | nd | nd | nd |
| | | **Non-Esterified** | |
| **Sample** | **FA** | **di-FA** | **tri-FA** |
| | | **µg/mg Sample (Dry Weight)** | |
| AX | Nd | nd | nd |
| AX-f | 1.14 ± 0.06 | nd | nd |
| Control | nd | nd | nd |

nd: not detected. AX: arabinoxylan; AX-f: fermented AX; Control: (no added carbon source).

The structural features of AX, such as the A/X ratio and the presence of phenolic acids, play a significant role in the capacity of bacteria to degrade these polysaccharides [15,33]. It has been reported that AX presenting a low A/X ratio can be more efficiently fermented than those that are highly substituted because xylanases prefer unsubstituted xylose regions, and most arabinofuranosidases act on monosubstituted xylose residues [33]. It has been also reported that AX with a low di-substituted xylan backbone is fermented relatively quickly by a porcine fecal microbiota [34]. Moreover, the presence of FA-esterified arabinose can decrease the fermentability of AXOS, as it limits arabinofuranosidase activity [15]. The AX used in the current study presented an A/X ratio of 1.1; an average molecular weight of 209 kDa; and FA, di-FA, and tri-FA contents of 5.45, 0.35, and 0.03 µg/mg polysaccharide, respectively [26], which are in the range reported for other maize AX [16,35]. Rascón-Chu et al. [36] reported the complete degradation of maize AX registering an A/X ratio of 0.85, a FA content of 0.34 µg/mg polysaccharide, and a Mw of 270 kDa by using a mixture of *B. ovatus* and *B. longum*.

### 3.5. SCFA Production

The production of SCFA after the in vitro fermentation of AX by the bacterial mixture is shown in Figure 5. AX fermentation produced a high concentration of total SCFA (277 µmol/50 mg AX), which is in accordance with the low pH value detected in the culture broth. This value is in the range reported by Kaur et al. [37] in maize AX (115–495 µmol/50 mg AX) during in vitro fecal fermentation. This high SCFA production indicates that the bacteria efficiently utilized AX as a sole carbon source, which was also evidenced by their growth in the culture medium (Figure 2). It has been reported that the growth of bifidobacteria in AXOS correlated with an increase in acetate concentration and a low pH [14]. The SCFA concentrations followed the order of acetate > propionate > butyrate. Acetic acid has been reported as the dominant SCFA produced during the in vitro fermentation of AX by bifidobacteria, followed by propionic and butyric acids as the less abundant metabolites [19]. The high concentration of acetate could be related to

the stimulation of bifidobacteria species by AX since it is the primary fermentation product of these microorganisms [13]. Production of propionic acid has been related to an increase in the *Bacteroides* population [38]. The beneficial effects of SCFA concerning their protective effect against colon carcinogenesis development have been widely reported [39–41]. In particular, butyrate has been demonstrated to show chemopreventive properties due to its anti-inflammatory and immunomodulatory effects in the colonic region [39,40,42]. Additionally, acetic acid, which is the most abundant SCFA produced during the fermentation of AX, has anti-inflammatory properties [43]. Butyric and acetic acids were demonstrated to exert antiproliferative effects against human colorectal cancer cells (DLD-1 cell line) by inhibiting the expression of genes encoding proteins involved in DNA replication and cell cycle/proliferation [5]. In addition, acetic acid induces apoptosis in colorectal cancer cells via lysosomal membrane permeabilization and the subsequent release of cathepsin D during apoptosis [6].

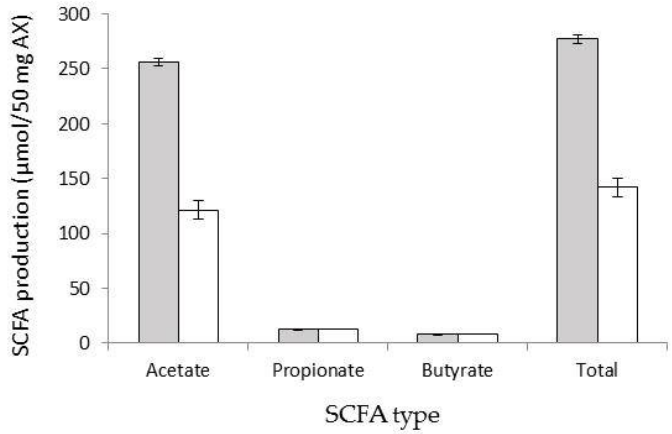


**Figure 5.** Short-chain fatty acids (SCFA) produced during in vitro fermentation (48 h) of AX (■) and Control (□, no added carbon source) using colonic bacteria. Each value is expressed as mean ± SD.

### *3.6. AX and AX-f Antiproliferative Activity on Caco-2 Colon Cancer Cells*

A previous study evaluated the effect of wheat AX fermentation supernatant (using human feces to ferment AX) on the proliferation of HT-29 colon cancer cells [7]. Nonetheless, the current is the first work reporting the effect of fermented AX from DDGS (using the specific bacteria bifidobacteria and bacteroides) on Caco-2 colon cancer cell proliferation. Figure 6 presents the proliferation percentage of Caco-2 cells exposed to different concentrations (0–1000 µg/mL) of AX and AX-f. The results indicate that the growth of Caco-2 cells was efficiently affected by all concentrations of AX-f. Interestingly, AX-f presented a dose-dependent inverse effect, showing a higher antiproliferative effect at lower concentrations. AX-f decreased Caco-2 cell proliferation from 80 to 40% when used at concentrations from 125 to 1000 µg/mL. In a previous study, Zhang et al. [44] found that at low concentration range (5–50 µg/mL), low molecular weight maize AX (0.1–10 kDa) presents more effective immune function. These authors suggested that direct interaction of fibers with colonic cells improves cytokine production, leading to antitumor effects. In the present study, the AX-f dose-dependent inverse effect could also be related to the viscosity in the culture medium due to the polysaccharide concentration. It has been reported that AX presenting a low Mw (60 kDa) can register a high intrinsic viscosity ([183 mL/g]) [45]. Higher concentrations of AX result in relatively highly viscous solutions [1], which could decrease the interaction between the metabolites and the cells, and then impact the antiproliferative effect of this polysaccharide.

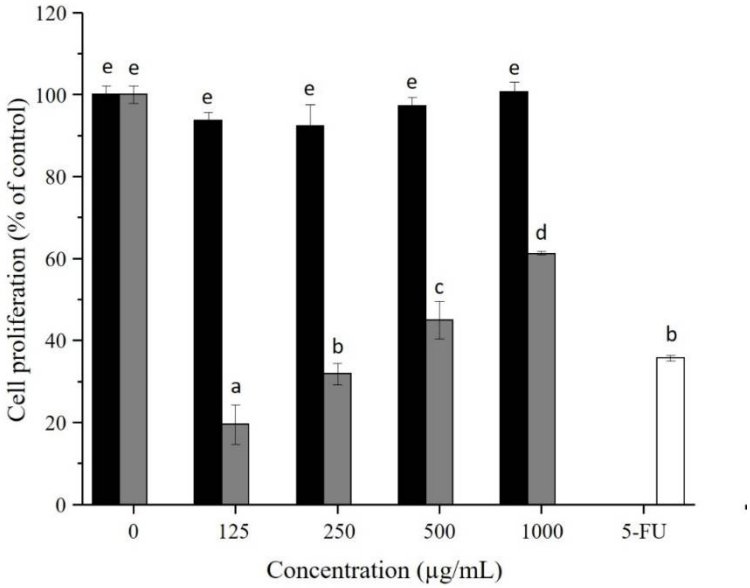


**Figure 6.** Effect of AX (■) and AX-f (■) on the proliferation of Caco-2 cells. 5-FU (□, 26 μg/mL) was used as positive control. Cells were incubated for 48 h at 37 °C before cell proliferation was measured. Untreated group: concentration = 0. Bars marked by unlike letters are significantly different at $p < 0.05$, according to a Tukey–Kramer test.

Previous research found that a concentration of 25% (*v*/*v*) of wheat AX fermentation supernatant inhibited the proliferation of the cell line HT-29 by 75%; however, the structural and physicochemical characteristics of the AX used by those authors were not reported [7]. It is possible that this dissimilarity in the antiproliferative effect of fermented AX could be related to the use of human feces to degrade AX and to the polysaccharide structural characteristics (molecular weight; FA, di-FA, and tri-FA contents; and arabinose substitution degree, among others). In general, maize AX presents a more complex structure, especially high FA and di-FA contents, than that of wheat AX, which could limit polysaccharide fermentation [12]. Particularly, the AX used in the previous study [7] reported no measurable amounts of FA or other hydroxycinnamic acids in its structure, in contrast to the one used in the present study (Table 1). A deferulated or uncross-linked AX structure can be degraded more easily by bacteria than a highly ferulated and cross-linked structure [12]. In addition, the AX fermented with the human gut microbiota was exposed to a wide variety of bacterial species [7], while only three pure cultures were used in the present study. Therefore, complete degradation of the AX could result in the production of oligosaccharides or carbohydrate residues that could be fermented easily, thus reducing the viscosity of the culture medium and favoring the interaction between metabolites and cells. The authors of the previous work also suggested that SCFA were mainly responsible for the antiproliferative activity of the fermented AX [7]. They reported a total SCFA concentration of 86 mM generated from AX fermentation, with acetic acid showing the highest concentration (54 mM) [7]. In the current study, the total SCFA concentration was 28 mM, with acetic acid being the most abundant at a concentration of 26 mM. However, the AX used in that study was fermented using human feces, which increased the concentration of SCFA produced due to the complex microbiota composition. In the present study, the total SCFA content in the cell proliferation test decreased from 345 to 43 μg/mL when the AX-f concentration used decreased from 1 to 0.125 mg/mL. In the literature, similar SCFA contents have been reported to present antiproliferative effects [46]. Some other metabolites produced during AX fermentation could contribute to the antiproliferative activity of the AX-f. The properties of phenolic acids against colon cancer have been widely documented [11], particularly the antiproliferative activity of FA against the colon cancer cell line Caco-2 through its intervention in different phases of the cell cycle [9,10]. It is

important to note that in the current investigation, free FA (non-esterified to AX) and esterified FA were detected in the AX-f (Table 1). Therefore, the free and esterified FA content in the proliferation tests diminished from 1.14 to 0.14 and from 2.07 to 0.26 μg/mL, respectively, when the AX-f concentration used decreased from 1 to 0.125 mg/mL. In the literature, similar FA contents have been reported to present antiproliferative effects [47]. In the present study, the FA (free and esterified) could be responsible of the antiproliferative effect of the AX-f beside the SCFA concentration.

Non-fermented AX was also tested against the Caco-2 cell line as previously reported [26]. The results showed that non-fermented AX slightly inhibits the proliferation of the human colon cancer cell line Caco-2 up to 8% (Figure 6). Samuelsen et al. [23] reported a similar behavior, in which different concentrations (0.5 to 3 mg/mL) of AX from barley did not have a significant effect on the proliferation of the cell lines HT-29 and Caco-2. Additionally, AX from DDGS has shown good biocompatibility with normal human colon cells (CCD 841 CoN), as reported elsewhere [26]. In the present study, the drug 5-FU, which was used as a positive control in the experiments, showed an evident antiproliferative effect on the cancerous human colon cell at the concentration used (26 μg/mL) (Figure 6). 5-FU is an antimetabolite drug that is widely used in the treatment of a range of cancers, particularly colorectal cancer. This agent exerts its anticancer effects through the inhibition of DNA synthesis due to the incorporation of its metabolites into RNA and DNA [48].

## 4. Conclusions

Ferulated AX recovered from the maize bioethanol industry can be efficiently fermented by the colonic bacterial mixture of *Bifidobacterium longum*, *Bifidobacterium adolescentis*, and *Bacteroides ovatus*, and the generated product (AX-f) inhibits the proliferation of Caco-2 cells up to 80%. Non-fermented AX only inhibits the proliferation of this colon cancer cell line by 8%. The results suggest that the AX-f dose range, the SCFA production, and the ferulic acid content are key determinants of antiproliferative activity in AX-f. These findings highlight the potential of fermented AX recovered from distiller's dried grains with solubles (DDGS) as an antiproliferative agent. However, in vivo studies are necessary to investigate the fermentation of this polysaccharide by the gut microbiota as well as the production of metabolites and their antiproliferative activity in the colonic region.

**Author Contributions:** Conceptualization, E.C.-M. and M.A.M.-E.; methodology, M.A.M.-E.; software, M.A.M.-E.; validation, E.C.-M., D.E.V.-R., H.A.-G., V.M. and A.R.-C.; formal analysis, M.A.M.-E., E.C.-M., D.E.V.-R., H.A.-G., V.M. and A.R.-C.; investigation, E.C.-M. and M.A.M.-E.; resources, E.C.-M., D.E.V.-R. and A.R.-C.; data curation, E.C.-M., D.E.V.-R., H.A.-G., V.M. and A.R.-C.; writing—original draft preparation, M.A.M.-E.; writing—review and editing, D.E.V.-R., E.C.-M., H.A.-G., V.M. and A.R.-C.; visualization, E.C.-M. and M.A.M.-E.; supervision, E.C.-M.; project administration, E.C.-M.; funding acquisition, E.C.-M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was funded by the "Fund to support research on the Sonora-Arizona region 2019", Mexico [Grant: 20614 to E. Carvajal-Millan].

**Acknowledgments:** The authors are delighted to acknowledge Alma C. Campa-Mada, Karla G. Martínez-Robinson, and Jorge Marquez-Escalante (CIAD) for technical support.

**Conflicts of Interest:** The authors declare no conflict of interest.

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