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Abstract: Background: Prebiotics are non-digestible compounds that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of resident colonic bacteria in the gut. Reported beneficial effects of prebiotics include reduced gut infections, better absorption of minerals, and notably, antitumorigenic effects. Arabinoxylan (AX)-oligosaccharides (AXOS) have been suggested to exert prebiotic effects in the gut, but their effect on colon carcinogenesis has not been studied so far. Aim of the study: To test the effect of AXOS in a rat colon carcinogenesis model. Methods: We determined the occurrence of two types of preneoplastic lesions [aberrant crypt foci (ACF) and mucin depleted foci (MDF)] in the colon of rats treated with the colon carcinogen 1,2-dimethylhydrazine (DMH) and fed either a control diet or a diet containing AXOS (4.8% w/w). Results. MDF counts were significantly lower in the entire colon of AXOS fed rats (MDF/colon were 7.5 ± 0.6 and 5.5 ± 0.6 , in Control and AXOS groups, respectively, means \pm SE, $p < 0.05$). Although the number of ACF in the entire colon was not significantly different between Control and AXOS fed rats, AXOS fed rats had significantly fewer ACF in the distal part of the colon than Control group rats (ACF/distal colon were 135.5 ± 15 and 84.4 ± 11 , in Control and AXOS groups, respectively, means \pm SE, $p < 0.05$). Conclusions: The present study shows that dietary intake of AXOS by rats reduces the occurrence of two types of preneoplastic lesions, thus suggesting a chemopreventive effect on colon carcinogenesis that should be confirmed in a long-term carcinogenesis experiment.

Arabinoxylan oligosaccharides (AXOS) reduce preneoplastic lesions in the colon of rats treated with 1,2-dimethylhydrazine (DMH)

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Summary

Background: Prebiotics are non-digestible compounds that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of resident colonic bacteria in the gut. Reported beneficial effects of prebiotics include reduced gut infections, better absorption of minerals, and notably, antitumorigenic effects. Arabinoxylan (AX)-oligosaccharides (AXOS) have been suggested to exert prebiotic effects in the gut, but their effect on colon carcinogenesis has not been studied so far. *Aim of the study:* To test the effect of AXOS in a rat colon carcinogenesis model. *Methods:* We determined the occurrence of two types of preneoplastic lesions [aberrant crypt foci (ACF) and mucin depleted foci (MDF)] in the colon of rats treated with the colon carcinogen 1,2-dimethylhydrazine (DMH) and fed either a control diet or a diet containing AXOS (4.8% w/w). *Results.* MDF counts were significantly lower in the entire colon of AXOS fed rats (MDF/colon were 7.5 ± 0.6 and 5.5 ± 0.6 , in Control and AXOS groups, respectively, means \pm SE, $p < 0.05$). Although the number of ACF in the entire colon was not significantly different between Control and AXOS fed rats, AXOS fed rats had significantly fewer ACF in the distal part of the colon than Control group rats (ACF/distal colon were 135.5 ± 15 and 84.4 ± 11 , in Control and AXOS groups, respectively, means \pm SE, $p < 0.05$). *Conclusions:* The present study shows that dietary intake of AXOS by rats reduces the occurrence of two types of preneoplastic lesions, thus suggesting a chemopreventive effect on colon carcinogenesis that should be confirmed in a long-term carcinogenesis experiment.

Introduction

Arabinoxylan (AX), formerly also referred to as pentosan, is a major constituent of cereal cell walls and consists of a backbone of β -D-xylopyranosyl residues (xylose), some of which are mono- or disubstituted with α -L-arabinofuranosyl residues (arabinose) [1, 9, 24]. From a dietary point of view, AX has raised interest as some types of oligosaccharides derived from it by enzymatic or chemical cleavage have antioxidant properties, due to the presence of hydroxycinnamic acids, mainly ferulic acid linked through ester bonds to the arabinose residues of AX [1, 25, 29, 40]. Moreover, arabinoxylan-oligosaccharides (AXOS) have also been suggested to exert a prebiotic effect in the gut [10, 12, 38]. Prebiotics are non-digestible compounds that beneficially affect the host by stimulating the growth and/or activity of one or a limited number of resident colonic bacteria in the gut [34, 37]. Among the beneficial effects of prebiotics, particular attention has been paid to their purported effect on colon cancer. Several experimental studies indicate that prebiotics, such as fructooligosaccharides or xylooligosaccharides (XOS), are protective against colon carcinogenesis [23, 37]. However, the effect of AXOS on colon carcinogenesis has not been studied so far. Therefore, in the present study we tested the effect of AXOS on colon carcinogenesis determining preneoplastic lesions [aberrant crypt foci, (ACF) and mucin depleted foci (MDF)] [7, 11] in rats treated with the colon carcinogen 1,2-dimethylhydrazine (DMH), a widely used experimental model to test the potential chemopreventive activity of food components [11].

Materials and Methods

Materials

1,2-dimethylhydrazine (DMH) was purchased from Sigma (Milan, Italy). Dietary components for the preparation of the AIN76 diet (see below) were purchased from Piccioni (Gessate, Milan, Italy). The AXOS preparation used in this study was obtained by xylanolytic hydrolysis of wheat bran, as described by Swennen et al. [35] and was provided by Fugeia NV (Leuven, Belgium). Table I presents the characteristics of the AXOS preparation, which had an

average degree of polymerisation (avDP) of 5 and an average degree of arabinose substitution (avDAS) of 0.21. Total, reducing end, and free saccharide contents were determined by gas chromatographic analysis [13] and calculation of AXOS content, avDP and avDAS was done as described earlier [13], except that no correction for arabinogalactan content was made due to low galactose contents. Moisture and ash contents of the preparation were analyzed according to AACCI methods 44-19 and 08-01, respectively [2]. Protein content was determined according to a Dumas combustion method, using an automated Dumas protein analysis system (EAS varioMax N/CN, Elt, Gouda, The Netherlands), an adaptation of the AOAC Official Method for protein determination [3] and using 6.25 as the nitrogen protein conversion factor. Ferulic acid (FA) content was determined by High Performance Liquid Chromatography with UV light detection at 310 nm according to Hartmann et al. [22], using o-coumaric acid as an internal standard. Total FA content is determined after saponification of the sample in 2M NaOH for 18h at room temperature under nitrogen atmosphere. Free FA is determined without prior saponification. Glucuronic acid (GluA) content is determined colorimetrically by the m-phenylphenol method using D-glucuronic acid for calibration [5].

Animals and treatments

We used 5-6-week old male F344 rats (Nossan, Correzzana, Milan, Italy), which were randomly allocated to two groups (15 rats per group). A control group (Control) was fed a high-fat diet based on the AIN76 diet [20], but with a higher portion of lipids (230 g/kg corn oil) and a lower level of cellulose (20 g/kg). The source of carbohydrates in this diet was sucrose (341 g/kg), maize starch (60 g/kg) and maltodextrins (Maldex 150, Syral, 60 g/kg). A treatment group (AXOS) was fed the same diet as the Control group, but this diet contained 60 g/kg of AXOS preparation instead of maltodextrins, providing a pure AXOS content of 48 g/kg. Ten days after the beginning of the experimental diets, both groups were treated twice, one week apart, with 100 mg/kg of DMH by subcutaneous injection. Dietary treatment (*ad libitum* for both groups) continued until the end of

the experiment, *i.e.* thirteen weeks after the first injection with DMH, when rats were sacrificed with CO₂ asphyxiation.

Determination of ACF and MDF

At sacrifice, the cecum and colon were dissected and washed with saline. The cecal contents and cecal walls were weighed. The colon was pinned flat on a polystyrene board to reduce any folding of the colon mucosa which would otherwise have interfered with a good visualization of MDF and fixed in buffered formalin [20]. The number of ACF and their multiplicity (number of aberrant crypts forming each ACF) were determined after methylene blue staining [4]. After ACF determination, methylene blue stained colons were kept in formalin and then processed with the high-iron diamine Alcian blue staining to determine the number of MDF and their multiplicity (number of crypts forming each MDF) [7]. Regarding the localization of ACF and MDF along the colon, the entire colon, extending from the anal to the cecal ends, was divided in two equally long parts: one proximal and one distal.

Statistical analysis

Data obtained from individual rats in the two groups were analyzed with t-test (unpaired samples) calculating the contrasts between means. Differences were considered statistically significant when p was <0.05 .

Results

Body weight and cecal parameters

There were no differences in body weights between the two experimental groups. Body weight at the beginning of the experiment was 159 ± 2.4 and 158 ± 2.4 (g) in Control and AXOS groups, respectively, and 357 ± 6.5 and 350 ± 6.5 (g) at the end of the experiment (means \pm SE).

Cecal content at sacrifice was significantly higher in the AXOS than in the Control groups, while the weight of the cecal wall did not differ between groups (Table II).

Preneoplastic lesions: aberrant crypt foci (ACF) and mucin depleted foci (MDF) determination

The number of ACF determined in the entire colon of rats was not different between Control and AXOS fed rats (Fig. 1, panel a). Similarly, the multiplicity of these lesions, i.e. the number of crypts forming each ACF, did not vary (crypts/ACF were 2.6 ± 0.1 and 2.7 ± 0.1 in Control and AXOS groups, respectively, means \pm SE). Interestingly, when considering the distribution of ACF along the colon, we observed that in the distal part of the colon, the number of ACF in the AXOS group was significantly lower than that in the Control group (Fig. 1, panel b). No difference between the two groups was observed for the proximal part of the colon.

The number of MDF in the entire colon was significantly lower in the animals fed AXOS than in the Control group (Fig. 1, panel c). The multiplicity of MDF was slightly lower in the AXOS group, but this effect did not attain statistical significance (crypts/MDF 13.2 ± 4.2 and 8.7 ± 2.2 in the Control and AXOS fed groups, respectively, means \pm SE). Considering the distribution of MDF along the colon in the two groups, we also found, as observed for ACF, a statistically significant effect in the distal part of the colon (Fig. 1, panel d).

Discussion

In the gut, prebiotics cause changes in the intestinal microbiota which have been associated with improved overall health, reduced gut infections, better absorption of minerals, and, notably, antitumorigenic effects [21, 28, 34]. Arabinoxylan oligosaccharides (AXOS) have been suggested to exert prebiotic effects in various experimental settings [12, 14, 15, 36]. In rats, AXOS with an average degree of polymerization of 5, as in the present study, increased acetate and butyrate concentrations in the colon, reduced intestinal protein fermentation and increased concentrations of cecal bifidobacteria [36]. In chickens, AXOS increased cecal bifidobacteria while reducing potentially pathogenic *Salmonella* strains [12, 14, 38,]. Moreover, addition of AXOS to the diet of healthy human volunteers caused a significant reduction of urinary ammonia excretion and higher

excretion via the feces, thus suggesting beneficial effects [10]. Given the earlier reported beneficial effects of AXOS, we were interested in studying their possible protective effect on colon carcinogenesis. Although tumours are the best endpoints for evaluation of the chemopreventive effects of dietary agents, preneoplastic lesions, if well correlated with carcinogenesis, can be used as cancer endpoints avoiding long-term carcinogenesis experiments, which are time and animal consuming [11]. The easy identification of aberrant crypt foci (ACF) and mucin depleted foci (MDF) in short-term studies makes these lesions very useful for testing potential chemopreventive efficacy of dietary components. ACF have been extensively characterized and demonstrated to show many features of preneoplastic lesions [11, 33]. In many chemopreventive studies, ACF showed a good correlation with long-term carcinogenesis experiments [33], although some studies also documented a disagreement between ACF and tumours, probably due to the heterogeneous nature of ACF [30]. MDF have been more recently introduced as cancer biomarkers [7, 18, 31, 32]. These dysplastic lesions carry alterations in the Wnt signalling pathway and mutations in the β -catenin, *Apc* and *K-ras* genes [16, 19]. Notably, we recently identified MDF in humans at high risk for colon cancer, a result indicating the relevance of these lesions in early colon cancer development [35]. Although MDF have been used so far in a limited number of studies [18, 20, 31, 32], they have shown a good correlation with carcinogenesis, making them promising biomarkers for the study of chemopreventive agents in colon carcinogenesis.

The results of the present study clearly show a significant effect of AXOS in reducing the number of MDF, early precancerous lesions of colon carcinogenesis. The effect of AXOS on MDF was evident in the entire colon, with marked effects for the distal part. Moreover, the MDF in the AXOS group tended to be smaller, as demonstrated by the lower multiplicity, but this effect did not attain statistical significance. Contrary to the clear-cut effect on MDF, the results on ACF showed that, while the number of ACF in the entire colon was not impacted by AXOS, there was a statistically significant reduction in the distal part of the colon. In relation to this result, it is interesting to note that the effect of dietary variations on colon cancer has been suggested to be

more evident in the distal than in the proximal colon [26, 27]. Also, in a previous study by Hsu et al. [23], in which XOS, structurally similar to AXOS, and fructooligosaccharides were found to reduce ACF, the enumeration of these lesions was carried out only in the distal part of the colon, while no data were presented for either the proximal or the entire colon.

Addition of AXOS to the diet did not affect the body weight of the animals, consistent with an earlier study [36]. The marked increase in cecal content caused by AXOS addition to the diet has been observed before for other prebiotics such as XOS, FOS, and inulin [6,8,23, 39], and is considered to be a normal physiological response to the presence of high concentrations of fermentable non-digestible oligosaccharides in the diet [6].

In conclusion, we showed a protective effect of AXOS on two types of preneoplastic lesions in a rat colon carcinogenesis model. We believe that this result is indicative of a possible chemopreventive effect of AXOS on colon carcinogenesis that should be confirmed in a long-term carcinogenesis experiment.

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Conflict of interests: WFB, CMC, IEJAF, and JAD hold stock (options) of Fugeia NV. WFB and JAD are directors of Fugeia NV.

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Table I: Characterization of the AXOS preparation

Compositional parameter	AXOS preparation
Moisture (%)	3.0
AXOS ^a (% dm) ^b	83.4
avDAS ^c of AXOS: 0.21	
avDP ^d of AXOS: 5	
Ferulic acid bound to AXOS ^e (% dm)	1.5
Glucuronic acid bound to AXOS ^f (% dm)	1.0
Glucose as part of poly/oligosaccharides ^g (% dm)	12.4
Galactose as part of poly/oligosaccharides ^g (% dm)	0.6
Mannose as part of poly/oligosaccharides ^g (% dm)	0.2
Free monosaccharides ^h (% dm)	1.6
Protein	0.4
Ash (% dm)	0.5

^a AXOS content = $0.88 \times [\text{arabinose}_{\text{TOT}} - \text{arabinose}_{\text{FREE}}] + [\text{xylose}_{\text{RED}} - \text{xylose}_{\text{FREE}}] + 0.88 \times [\text{xylose}_{\text{TOT}} - \text{xylose}_{\text{RED}}]$. ^b dm= dry matter. ^c avDAS = $[\text{arabinose}_{\text{TOT}} - \text{arabinose}_{\text{FREE}}] / [\text{xylose}_{\text{TOT}} - \text{xylose}_{\text{FREE}}]$. ^d avDP = $\{[\text{arabinose}_{\text{TOT}} - \text{arabinose}_{\text{FREE}}] + [\text{xylose}_{\text{TOT}} - \text{xylose}_{\text{FREE}}]\} / [\text{xylose}_{\text{RED}} - \text{xylose}_{\text{FREE}}]$. ^e bound ferulic acid = $0.907 \times [\text{ferulic acid}_{\text{TOT}} - \text{ferulic acid}_{\text{FREE}}]$; free ferulic acid content < 0.01 % dm. ^f bound glucuronic acid = $0.907 \times [\text{glucuronic acid}_{\text{TOT}}]$; free glucuronic acid content < 0.01 % dm. ^g glucose content as part of poly/oligosaccharides = $0.9 \times [\text{glucose}_{\text{TOT}} - \text{glucose}_{\text{FREE}}] + [\text{glucose}_{\text{RED}} - \text{glucose}_{\text{FREE}}]$; mannose and galactose as part of poly/oligosaccharides are calculated in the same way. Total poly/oligosaccharides is the sum of AXOS, ferulic acid and glucuronic acid bound to AXOS, glucose-, galactose- and mannose as part of poly/oligosaccharides. ^h free monosaccharides = $\text{glucose}_{\text{FREE}} + \text{xylose}_{\text{FREE}} + \text{arabinose}_{\text{FREE}}$. Other free monosaccharides are < 0.05% dm.

Table II: Cecal parameters in rats treated with Control or AXOS diet

	Control	AXOS
Cecal content (wet weight, g)	1.9±0.2	3.2±0.3 **
Cecal wall (wet weight, g)	1.2±0.1	1.5±0.1

Values represent means±SE n=12, since this determination was carried out in a random subgroup of rats for each group. **: significantly different when compared with Control group (p<0.001).

LEGEND TO THE FIGURE

Figure 1. Number of ACF and MDF in the colon of Control (grey bars) or AXOS (white bars) group rats. Panels a and c: number of ACF and MDF, respectively, in the entire colon; panels b and d: number of ACF and MDF, respectively, in the proximal and distal colon of the two experimental groups. *: significantly different (p<0.05) when compared with Control group. Bars are means + SE, n=15.

