



## **Bifidogenic effect of a wheat arbinoxylan (MC-AX) is observed across two animal models, a stimulated human intestine model (SHIMES®) and a clinical study.**

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Bifidogenic effect of a wheat arabinoxylan (MC-AX) is observed across two animal models, a simulated human intestine model (SHIME®) and a clinical study.

## Abstract

Dietary fibres consist of carbohydrate polymers of plant origin and are neither digested nor absorbed in the small intestine. They have at least one functional property linked to stools production, colonic fermentation, cholesterol, glucose or insulin levels. Different models are available to investigate the prebiotic potential of such a molecule. The aim of this study was to compare our clinical data with three previously unpublished preclinical studies, all looking for prebiotic activity of a medium-chain arabinoxylan (MC-AX) extracted from wheat endosperm. MC-AX was tested in an in vitro multicompartment digestive system (SHIME®), piglet and rat models as well as a clinical study, at doses ranging from 1 to 10g/day and exposed for 2 to 4 weeks. Selected bacterial counts and metabolites were measured at different time points. MC-AX induced an increase in Bifidobacterium in treated groups compared to placebo of at least one log unit in all models. This could be measured as early as 7 days (SHIME®) with the strongest variation observed in piglets (2 logs) at day 14. We observed a significant decrease in acetate production, balanced by an increase in both propionate and butyrate in the rodent and human studies. While there are some quantitative differences between the models, partially due to dose or species specificities, there is a clear and consistent overall pattern. Data were also compared to published clinical trials on dietary fibres to explain some structure-activity relationships relative to their physicochemical properties.

Keywords: xylooligosaccharide, SHIME®, rodent, piglet, clinical trial

## Introduction

Dietary fibres are carbohydrate polymers (average degree of polymerisation (avDP)  $\geq 3$ ) of plant origin, which may or may not be associated in the plant with lignin or other noncarbohydrate components (polyphenols, waxes, saponins, cutins, phytates, phytosterols, etc.) or, as is the case for the product described in this paper, a processed (by physical, enzymatic or chemical means) carbohydrate polymer (Elleuch et al. 2011; Phillips and Cui 2011; Lee and Prosky 1994). In addition dietary fibre is neither digested nor absorbed in the small intestine. To be considered prebiotics, those non-digestible food ingredients need to beneficially affect the host by selectively stimulating the growth and/or the activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health (Gibson and Roberfroid 1995; Gibson et al. 2004). It has at least one physiological effect (Lunn and Buttriss 2007) on stools production (Harvey, Pomare, and Heaton 1973; Kurasawa, Haack, and Marlett 2000), colonic fermentation, pre-prandial cholesterol levels (Marlett 1997; Pereira and Gibson 2002) or post-prandial blood sugar and /or insulin levels (Aleixandre and Miguel 2008; Hallfrisch and Behall 2000; Lu et al. 2004). Arabinoxylan (AX) is the main non-starch polysaccharide of cereal grains (Izydorczyk and Biliaderis 1995) and can be found in a variety of tissues of cereals such as wheat, rye, barley, oat, rice, sorghum, as well as in other plants (bamboo shoots, rye grass and pangola grass). These complex carbohydrates occur in cell walls of the starchy endosperm cells and the aleurone layer in most cereals (60-70% (w/w) of the total carbohydrate). They can be found in the endosperm cell walls of barley (20% (w/w)) and rice (40% (w/w)). Nonendospermic tissues of wheat, particularly the pericarp and testa, also contain a

very high concentration of AX (64% (w/w)) (Grootaert et al. 2007; Williams et al. 2011). Arabinoxylan consists of long backbone chains of anhydro-D-xylopyranosyl residues linked together by  $\beta$ -(1 $\rightarrow$ 4) glycosidic bonds. The major substituents are single  $\alpha$ -L-arabinofuranosyl residues attached at the 2- or 3-position to isolated xylosyl residues of the main chain, sometimes to two adjacent residues, less frequently to three, and not to four or more consecutive residues (Andrewartha, Phillips, and Stone 1979). Xylose can also be directly coupled with acetic acid or methylglucuronides (Van Craeyveld et al. 2008; Izydorczyk and Biliaderis 1995). Some of the arabinose units are substituted with the phenolic units such as ferulic acid or p-coumaric acid (Cleemput et al. 1993), which is linked with an ester bond between its carboxyl group and carbon 5 of the arabinose. Ferulic acid can bind different xylose polymeric chains together, creating a jellified, insoluble cross-linked structure (Van Craeyveld et al. 2008). The degree of arabinose substitution (DAS) refers to the arabinose moieties on the xylose backbone and is further described as arabinose/xylose ratio. AX from wheat, rye or barley tend to have a lower DAS than polymers from rice or sorghum (Maes and Delcour 2002). Similarly there are differences in substitution ratio depending on the tissue. AX from wheat endosperm has a DAS of 0.5 to 0.7 while aleurone and seed coat are less substituted at 0.1 to 0.4 and the outer pericarp is highly substituted at 1.1 to 1.3 (Broekaert et al. 2011; Izydorczyk and Biliaderis 1995). The substitution and distribution of side chains are thus important factors in the physicochemical properties of AX. As for other polymers, the degree of polymerization (DP), defined as the molecular weight of the polymer divided by the molecular weight of the repeating units, is an important factor in the physicochemical properties of AX (Andrewartha, Phillips, and Stone 1979). It is thought that cereals can have polymers constituted of 1500 to 15000 residues (Ebringerova and Heinze 2000) although it is quite difficult to determine for sure as degradation can occur early during the extraction process (Broekaert et al. 2011). Native AX from wheat endosperm could have a molecular mass up to 800.000g per mol, meaning that the degree of polymerization can reach 6000. Arabinoxylan oligosaccharides (AXOS) can be obtained by enzymatic hydrolysis. Depending on the nature and degree of substitutions, a number of microbial enzymes can be used. They can be found on the cereals themselves (Broekaert et al. 2011), added during food processing such as bread or beer making (Courtin et al. 2009), or ultimately they can be produced by colonic bacteria such as *Bacteroides* or *Roseburia* (Chassard et al. 2007). AX can be degraded by endo- $\beta$ -1,4-xylanases,  $\alpha$ -L-arabinofuranosidases,  $\beta$ -xylosidases,  $\alpha$ -glucuronidases or even ferulic acid esterases (Grootaert et al. 2007). Xylanases are the most obvious for AX degradation and cleave the xylose backbone randomly to shorter fragments (Courtin and Delcour 2002). As well as AXOS or short-chain arabinoxylan (SCAX), which are end-products characterized by DP 2-10, AX hydrolysate can be further divided into long-chain arabinoxylans (LCAX) with DP > 50, and medium-chain arabinoxylan (MC-AX) with intermediate DP values, depending on the degree of hydrolysis. Prebiotic activity of dietary fibres has long been investigated using different models. In vitro models, whether using pure bacterial cultures or faecal samples, whether using static or dynamic fermenters, are the first port of entry for prebiotic investigations. Rodents (mice and rats) are then often used as a primary in vivo tool before moving to clinical studies. Piglet, for its size and common physiological features is more and more considered as a pertinent model for investigations of the digestive tract with better extrapolation to human (Rowan et al. 1994). A quick survey of search engines Pubmed/Medline or ScienceDirect using keywords "oligosaccharides", "dietary fibre", "prebiotic" and "in vitro", "rodent", "pig" or "clinical trial" led to hundreds of hits. There is therefore little chance of original data, whatever the dietary fibre chosen. It is important however to note that each publication is using a given model but very few compare between models (Koecher et al. 2014). There is thus the question of whether all preclinical models are good predictors of human clinical outcomes. Medium chain arabinoxylans have been chosen to investigate this hypothesis. The aim of this work is therefore to compare data from our clinical study

(Lecerf et al. 2012) to previously unpublished preliminary tests on in vitro and animal models using the same active ingredient, MC-AX. 2. Materials and Methods

### Ethical statements

The clinical trial was conducted according to the guidelines laid down in the Declaration of Helsinki and French regulation. The study protocol (2007-A00273-50) was approved by the regional Ethics Committee for North West France based at the Lille University Hospital and registered on [clinicaltrials.gov](https://clinicaltrials.gov) (NCT02287844). Written informed consent was obtained from all subjects prior to inclusion. Animal studies were carried out in strict accordance with the recommendations in the European Union guidelines and regulations. The protocols for the rodent and piglet studies were approved by the committee on the Ethics of animal experiments of the University of Liege and of Institut polytechnique LaSalle Beauvais, respectively. All efforts were made to minimize animal suffering at any time.

**Experimental product MC-AX** The raw material is a by-product from wheat endosperm (DF3 SAS France). It was obtained from purification of the side stream of a wheat starch producing factory using a three phase decanter for the separation of the two main streams – starch and gluten. This side stream was purified in order to eliminate most of the starch, proteins, minerals, and fats, arabinoxylan was partially hydrolysed by means of an endoxylanase, and the reaction mixture was concentrated and spray dried. The obtained MC-AX sample, a white powder, was characterized by a dry matter of 95%, an AX content of 80% on DM, an avDP of 25 (range 7-300) and DAS 0.75.

### Experimental models

The products described above were tested on four different experimental models to investigate their bifidogenic activity. Only markers pertinent to the bifidogenic and prebiotic objectives are presented here. Similarly, rat and human studies included additional test groups that are not reported in this article. As statistical analyses were performed by individual comparison between test and placebo groups, this does not affect the 2.3.1. SHIME® study Description of the SHIME® model has been published elsewhere (Joly et al. 2013; Molly, van de Woestyne, and Verstraete 1993; Possemiers et al. 2004; Reygner et al. 2016). Briefly, stool from 5 healthy volunteers were pooled and slurry inoculated into the fermenters 4 to 6 corresponding to the colonic compartments of the system, whilst a specific liquid diet is initially added to fermenter 1 corresponding to the stomach to stimulate transient microflora in the whole system. Once the microbiota is stable, the fibre is added to the twice-daily basal nutriment meal at a final concentration of 10g/day. Samples from fermenters 2 to 6 were collected at baseline and after 7 and 15 days intervention under sterile conditions to measure pH as well as for bacterial quantification. A final set of samples was collected 15 days after the last fibre meal to assess the residual effect.

**Rat study** Weaned male Wistar rats (Charles River, Brussels) were housed individually in metabolic cages (22°C, 70% humidity, 12h light-cycle). Animals were assigned to placebo or test group (n=8 in each group) through randomized complete block design. Test fibre was fed at a concentration of 7.5% (6g/kg diet) in a semi-purified diet, replaced by 5% starch and 2.5% saccharose in the placebo diet. Initial and final weights of the animals were 108 and 237g, respectively and did not differ among experimental groups. The intake dry matter was limited to 12.6g per day based on the voluntary intake measured during and acclimation period of 5 days prior to the experiment. Twenty-four hour stool samples were collected on day 10 and 20 for bacterial quantification. On day 21, the rats were

killed at 5h post prandial by CO<sub>2</sub> overdose and caecal content were rapidly collected for pH and short chain fatty acid (SCFA) measurements.

**Piglet study** Long white F1 weaned male piglets were housed in two bands of 8 animals fed either the placebo or test diet (10g/kg diet). Randomization ensured that weight were homogenous in both groups at the beginning of the intervention. Diets (Trouw Nutrition, France) were produced as powder with energy content of 17% (w/w) proteins, 8% (w/w) lipids and 43% (w/w) carbohydrates. For energy balance, 7.5% MC-AX were replaced by 3% saccharose. Food intake did not vary between groups but weight gain was increased in treated versus placebo group ( $p=0.024$ ). No pathology nor variation in cleanliness of the animals were observed throughout the experimentation. Fresh stool samples were collected in each pen on day 1 and 14 of the intervention and thus represent the production of both animals present in that area.

**Human study** The complete protocol for this clinical study, including selection and exclusion criteria, has been published elsewhere (Lecerf et al. 2012). Briefly, healthy volunteers (18-24 years old; 20 per group) were fed either a placebo (maltodextrin) or test fibre (5g/day) in orange juice as a twice-daily meal supplement for 4 weeks. Randomization was stratified for homogeneous distribution of body mass index (BMI) and estimated fibre intake between groups. Stool samples were collected at 14 and 28 days of intervention for pH measurements, bacterial quantification and detection of SCFA. A range of additional markers were measured and are fully described in the study report and related publication.

#### Measure of biological parameters

**Bacterial count by microbiological techniques** For SHIME®, 5mL were taken from each reactor 3 to 6 at three times (Joly et al. 2013). Dilutions were plated on various selective and non-selective media for quantitative and qualitative cultures of aerobic and anaerobic microflora. 100 µL of each dilution was cultured on different plates shown table 1. All aerobes plates were incubated at 37°C for 48 hours. All anaerobic plates were incubated in anaerobic chamber (Bactron Anaerobic, Sheldon Manufacturing) and were examined after 4 days of incubation at 37°C. The micro organisms were identified using standard microbiologic techniques. Bacterial viable counts are expressed as log<sub>10</sub> ColonyForming-Units CFU/mL.

**Bacterial detection by qPCR** Specific bacterial quantification was conducted using an absolute real-time PCR method. Briefly, bacterial DNA was extracted using the Qiagen DNA stool Kit (Courtaboeuf, France). The 16s rRNA copy number DNA samples were quantified by PCR. Primers and fluorogenic probes are described elsewhere (Pouillart et al. 2010). Quantitative PCR was performed with 100 ng of DNA, 12.5 µL of 2x Taqman PCR master mix (Applied Biosystems), 300 nmol of each primer and a 200 nmol probe in a final volume of 25 µL. Thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C followed by 45 repeats of 15 s at 95°C, and 1 min at 60°C. A negative control containing distilled water was included.

**Quantification of lactate and short chain fatty acids** Lactate was measured on in vitro samples according to Talasniemi et al (Talasniemi et al. 2008) with some variants. Samples from the fermenters were diluted 4 fold in triethanolamine (TEA) buffer (0.1M, pH 9.15) and the supernatant incubated with trichloroacetate (TCA) buffer to precipitate protein residues before analysis. The quantification of D- and L-lactate was performed using the enzymatic Lactate assay kit (Sigma-Aldrich, France) with colorimetric detection. Volatile fatty acids were extracted and measured according to Scheppach et al. (Scheppach, Fabian, and Kasper 1987) with some variants. Two volumes sample were homogenized in 5 volumes ddH<sub>2</sub>O then centrifuged at 4500 g for 5 min. The supernatant was acidified to pH 2 with H<sub>2</sub>SO<sub>4</sub> (2 M) and injected into a BP21 gas chromatography

column (L: 30 m, id: 530  $\mu$ m, film: 1  $\mu$ m) along with an internal standard (4-hydroxy-4-methyl-2-pentanone). Hydrogen was supplied as the carrier gas at a flow rate of 1.5 ml/min. The initial oven temperature was 135°C and was kept there for 6 min, and then raised to 180°C by 25°C/min and held there for 1 min, then further increased to 230°C by 25°C/min, and finally held at 230°C for 1 min. Glass wool (Supelco) was inserted in the glass liner of the split injection port. The temperatures of the flame ionization detector (FID) and the injection port were 240 and 280°C, respectively. The flow rates of hydrogen and air as makeup gas were 40 and 400 ml/min, respectively. The injected sample volume for Gas Chromatography (AutoSystem XL, PerkinElmer, France) analysis was 1  $\mu$ l, and the running time for each analysis was about 10 min. The different SCFA were identified according to the retention time of the various elution peaks. Quantification was obtained by comparison to a standard curve. All data were reported to dry matter.

Data and statistical handling Depending on the model, sample size or sample distribution for the specific markers, parametric or non-parametric tests were used to compare placebo and test groups. Additional statistical tests are described in the result section whenever applicable. All statistical tests were performed using SPSS v17.0 for Windows (SPSS Inc). All data are presented as means and standard deviations whenever possible. 3.

## Results

### Modulation of bacterial populations

Lactic acid bacteria. One parameter that was included in all four models is quantification of Bifidobacterium. Data from animal and clinical studies were obtained from quantitative real time PCR, while data from the SHIME® model were obtained by culture of live bacteria in selective media. In addition primer sequences were different between models; therefore quantitative data cannot be compared from one species to another. It is however possible to compare qualitative data and trends. All data are plotted in figure 3 and we can observe that AXOS induced an increase in Bifidobacterium in treated groups of at least one log unit, thus highly significant in terms of bacterial modulation. This could be measured as early as 7 days in the treatment (SHIME®, Figure 2). In the rodent model, the difference is clinically pertinent at one log unit against the baseline values for the treatment group at both 10 and 20 days, but variation from placebo was weaker at 0.7 and 0.2 log, respectively (Figure 2). The strongest variation was observed in piglets with nearly 2 log increase in 14 days (Figure 2). Lactobacillus variation was also measured but we observed different patterns for these bacteria. There was no variation between placebo and treatment or over time in the rodent and human studies (Figure 3). An increase of 0.7 and 1.6 log were observed after two weeks in the piglet and SHIME® models, respectively (Figure 3). Different sub-species or distribution of Lactobacillus may be present in ecosystem from the different models, inducing a selective response.

Other bacteria. While Bifidobacterium and Lactobacillus increased in the fermenters, Enterococcus/Streptococcus was stable in the SHIME® model with a possible transient increase in the 4th fermenter (proximal colon) at 7 days. Peptostreptococcus was measured in the clinical study and showed a transient increase at 14 but not 28 days. No data is available from the rat study. Bacteroides and Clostridium did not significantly vary in the SHIME® model. They did not vary significantly from the placebo group in the clinical trial either. They were not measures in the animal models.

### Carbohydrate metabolism.

Short chain fatty acids were measured in caecum content of the rodent and in the faeces of human. Figure 4 represents distribution profiles of acetate, propionate and butyrate in the placebo and treatment groups after 21 and 28 days in the rat and human, respectively (Figure 4). Baseline profiles in both groups were not significantly different, nor were they different from the placebo profile at the end of the study (data not shown). In both species, we observed a significant decrease in acetate production, balanced by an increase in both propionate and butyrate. Butyrate production may be favoured in human (+7%, Figure 4A) and rat (+15%, Figure 4) while propionate shows higher increase in the rat (+3%, Figure 4). The difference may also be linked to the sampling site, human measures being done on faeces while the rat measures are done on caecum content. Further degradation or crossfeeding may occur further down the digestive tract. Lactate production was measured in the SHIME® model and showed a time-dependent increase in all fermenters (Figure 4), although concentrations were higher in the first colonic section.

## Discussion

The object of this paper was to compare the bifidogenic and general prebiotic activity of a single molecule, MC-AX, over four different models and to determine whether the three preclinical studies are good predictors of human outcome. It is unusual to find for the same product complementary information from different models going from in vitro to clinical studies. This is the first time to our knowledge that four different models of prebiotic activity are being directly compared using the same dietary fibre as a reference product. The four studies were designed to investigate the prebiotic and bifidogenic effect of a dietary fibre with specific physicochemical properties. While they were designed and performed independently, it is interesting to compare and contrast the results so each model, with all its specificities, can add to the overall understanding of the prebiotic effect of MCAX. The first parameter that we can consider and which is identical on the four models is the age of the subjects. The animals selected were juvenile at the beginning of the studies, consistent with the target population from the clinical trial and SHIME® (young adults). Looking at other similarities, the in vitro SHIME® model and clinical study share a species, both investigating the human microbiota. On the other hand, animal studies represented global organisms where the interactions between microbiota and the intestinal tract could be taken into account, hence closer to the clinical setting. The samples collected were similar in the human and piglet studies (stool) while caecal and colonic content were collected from the rat and in vitro studies, respectively. Therefore, and despite species variations, a number of comparisons are possible. We will first compare the individual models with data extracted from the literature before moving to a comparison between models. We will conclude on what we learned from this exercise and whether preclinical models are transferable to human functions in the field of prebiotic activity.

## In vitro data

**Prebiotic effects** A number of publications, some of which cited in the introduction, have reported prebiotic effects of arabinoxylans in vitro. The main findings are summarised alongside our data in table 2. A number of information can be deduced from this table. The first one is an overall consistency between our data and published studies on the bifidogenic potential of arabinoxylans. Some structure-activity relationships can also impact the bifidogenic and prebiotic activities. Short-chain arabinoxylans (SC-AX) have a higher fermentation rate compared to longchain arabinoxylans (LC-AX). This is observed by a higher production rate of butyrate and propionate with SC-AX compared to LC-AX (Mäkeläinen et al. 2009). SC-AX are also associated with stimulation of the growth of both *Bifidobacterium* and *Lactobacillus* while LC-AX mostly impact *Bifidobacterium* in a

batch culture set-up (Hughes et al. 2007) but also semi-continuous model (Mäkeläinen et al. 2009). Our product, a medium-chain arabinoxylan (MC-AX) is in agreement with those data in terms of bacterial impact as *Bifidobacterium* is strongly stimulated and *Lactobacillus* is only slightly affected in our in vitro model. The degree of arabinose substitution (DAS) refers to the arabinose moieties on the xylose backbone and is further described as arabinose/xylose ratio. AX from wheat, rye or barley tend to have a lower DAS than polymers from rice or sorghum (Maes and Delcour 2002). Similarly there are differences in substitution ratio depending on the tissue. AX from wheat endosperm has a DAS of 0.5 to 0.7 while aleurone and seed coat are less substituted at 0.1 to 0.4 and the outer pericarp is highly substituted at 1.1 to 1.3 (Broekaert et al. 2011; Izydorczyk and Biliaderis 1995). It is difficult however to assess the exact role of DAS from those data as AX29/0.30 (Sanchez et al. 2009) and AX25/0.75 (us) are not obtained from the same concentration. However the role of arabinose in the *Lactobacillus* stimulation can be suggested when comparing short-term exposure with long-chain XOS (DP40) and AXOS (DP60), the first one inhibiting *Lactobacillus* growth (Mäkeläinen et al. 2009) while the second has no effect (Van den Abbeele et al. 2009). The substitution and distribution of side chains are thus important factors in the physicochemical properties of AX. Ferulic acid (F) linkage can also play a major role in the prebiotic effect of arabinoxylans. FAX have a lower fermentation rate, bacterial stimulation than free AX (Grootaert et al. 2007; Hopkins et al. 2003). Propionate production is also higher in free rather than Flinked AX (Hopkins et al. 2003) and some bacterial specificity seem to apply. In the MCS model, insoluble LC-F-AX were less efficient prebiotic than soluble free LC-AX (Vardakou et al. 2007). But xylanase-treated F-AX produced SC-F-AX that presented prebiotic and bifidogenic activity close to that of other reference oligosaccharides (Rycroft et al. 2001), again demonstrating that the degree of polymerization may play a more important role than substitutions. Both SC-AX and LC-AX, when compared to inulin, presented a prebiotic activity that was displaced toward distal compartments of the colon (Grootaert et al. 2007; Van den Abbeele et al. 2013), in agreement with our findings on MC-AX. Results from van den Abbeele et al (Van den Abbeele et al. 2013) on LC-AX indicate that the prebiotic effect such as stimulation of *Bifidobacterium* and *Lactobacillus* remains after 2 weeks wash-out of the SHIME® system. Our data suggest that the effect was reversible for MC-AX using the same model. In vitro batch culture studies are also a great tool to better understand the selectivity of bacterial strains to saccharolytic substrates. While the main outcome is an increase in the *Bifidobacterium* and *Lactobacillus* strains, these are not propionate or butyrate producers. And even though we did not detect any changes in these two metabolites, they are strongly associated with arabinoxylan fermentation. The growth promoting effect on selected *Lactobacillus* and *Bifidobacterium* strains has been reported by in vitro culture experiments (Grootaert et al. 2007; Kontula et al. 2000). Some *Bacteroides*/*Eubacterium* strains are known to produce propionate or butyrate from arabinoxylans, either directly or through cross feeding (Chassard et al. 2008; Chassard et al. 2007; Duncan et al. 2002). Lactate conversion to propionate by some of these strains is well known (Belenguer et al. 2007). While we did not observe a stimulation of the species, the PCR probes or culture media may not have been selective enough to target the butyrate producers. Similarly, lactate conversion to propionate or butyrate could not be proven from our data. Overall in vitro data obtained from either one of the 4 most prominent colonic simulation models suggest a bifidogenic activity of AX, more pronounced with shorter-chain and noncross-linked molecules. This may be associated with stimulation of *Lactobacillus* and some butyrate-producing bacteria. The main fermentation site is the distal colon and is expressed as increased propionate and butyrate production. These metabolites were not detected in significant amount in our in vitro study. They were however increased in the clinical study.

#### Animal models



Bifidogenic activity of AX (whether SC-AX, MC-AX or LC-AX) has been reported in vivo in different rodent models (Van Craeyveld et al. 2008; Hsu et al. 2004; Neyrinck et al. 2011; Van den Abbeele et al. 2011) and is a consistent benefit of arabinoxylan consumption. The major site of Bifidobacterium production seems dependent upon the degree of polymerisation, with higher DP shifting the fermentation towards the distal parts of the tractus (Van Craeyveld et al. 2008; Damen et al. 2011). Lactobacillus stimulation is not as clear. It was observed with XOS (Christensen et al. 2014; Santos, San Mauro, and Díaz 2006) but not with AXOS (Damen et al. 2011). The impact on SCFA production and distribution seems to be less consensual. Van Craeyveld (Van Craeyveld et al. 2008) and van den Abbeele (Van den Abbeele et al. 2011) have shown an increase in propionate production in the caecum. This is in agreement with observations on in vitro models presented previously (Grootaert et al. 2009; Van den Abbeele et al. 2013) where propionate was the main bacterial metabolite. Lu et al (Zhong X. Lu et al. 2000) suggest that acetate is produced in the caecum while propionate appears further along and accumulates in the faeces. In our study, it is butyrate that is mostly modulated, in agreement with in vitro observations (Hughes et al. 2007). Similarly to in vitro research, van Craeyveld investigated structure activity relationships (Van Craeyveld et al. 2008). The authors suggested that structure may impact which saccharolytic metabolites will be produced. Butyrate seems to increase as the degree of polymerisation decreased while propionate was associated with increased arabinose substitution. There also seems to be a role for the solubility of the fibre, which has not been taken into account in other models. Damen et al (Damen et al. 2011) showed that short-chain soluble arabinoxylans and long chain soluble arabinoxylan increased acetate production in the caecum, associated with lower pH and increased Bifidobacterium populations. On the other hand, long-chain insoluble arabinoxylan increased butyrate production and the butyrate producer Roseburia without affecting Bifidobacterium. Hence the structure-activity relationships work on a number of parameters and their interactions make it more difficult to predict the behaviour of a given molecule. Piglet model are less common but find similar increase in the production of propionate and acetate both in vitro (Wang et al. 2004; Williams et al. 2011) and in vivo using cannulated animals (Wang et al. 2004). Neither publication reported on bacterial variations. Each of the two animal studies also showed particularities. Rodents are more reproducible, due to their selected genetic background, while piglets are closer to the human intestinal properties (Rowan et al. 1994). The two models differ by the number of animals necessary and volume of collected samples, hence affecting the number of complementary analyses that can be done on the samples. Recently a model of polycannulated pig was developed, allowing for investigation on specific sections of the digestive tract (Nitravova et al. 2013). This will allow cumulating the benefits of the in vitro and in vivo systems. Overall, animal data from the literature are consistent with our findings and within vitro and clinical data reported herein.

#### Clinical data.

Human trials are by definition the optimal study design for human nutritional applications. Investigators also have the opportunity to work on large sample volumes and run a wide range of analyses. This was the case here and the full report can be found in a previous publication (Lecerf et al. 2012). We investigated at the same time a wide range of related biomarkers, going from the classical microbiota and carbohydrate metabolites to bacterial enzymatic activity, pH or protein putrefaction metabolites. This gave us more detailed information on the effect of the test product. Compared to other prebiotics like inulin, only a few clinical trials have been investigating the prebiotic activity of arabinoxylans. Matteuzzi et al (Matteuzzi et al. 2004) fed volunteers 10g/day of a prebiotic product containing up to 10% arabinoxylans and 10% raffinose for 3 weeks. No clear effect was observed. Post-hoc analyses of the results demonstrated that only for the individuals with a baseline level below a set threshold ( $8\log_{10}$  CFU/g and  $4.5\log_{10}$  CFU/g dry matter for

Bifidobacterium and Lactobacillus, respectively) was it possible to increase the population of lactic acid bacteria. No indication on the product could help define the nature of the arabinoxylans. A number of other studies reported the effect of xylans on healthy populations with doses ranging from roughly 2.5g/d (Damen et al. 2012; Finegold et al. 2014; Maki et al. 2012; Walton et al. 2012) to about 5g/d (François et al. 2014; Lecerf et al. 2012; Maki et al. 2012), 10g/d (Childs et al. 2014; Cloetens et al. 2010) and up to 30g/d (Windey et al. 2014). Bifidogenic activity was detected, although not to a clinical level of 1 log in 4 of the articles. Cloetens et al (Cloetens et al. 2010) fed volunteers 10g/day SC-AX (avDP=6; DAS=0.26) for 3 weeks. Bifidogenic activity was barely significant as the placebo product also induced bacterial growth. François et al (François et al. 2014) detected an increase in Bifidobacterium after 3 weeks of treatment at 5g/d with another SC-AX (avDP=5; DAS=0.19). Finegold et al (Finegold et al. 2014) did not show any stronger activity after 8 weeks treatment at 8g/d with XOS. Finally a dose of 4.8g/d for 3 weeks showed similar low bifidogenic activity (Maki et al. 2012). Another article using 2.4g/d AXOS produced in situ in white bread did not show any bifidogenic effect (Walton et al. 2012). This is all lower than our data, showing an increase over 1 log at a dose of 5g/d. One reason may be the structure of the arabinoxylan, our product MC-AX being a higher DP, the bacterial stimulation may be longer-lasting, in agreement with in vitro and preclinical findings. A stimulation of Lactobacillus is reported by only 2 articles (Cloetens et al. 2010; Walton et al. 2012), again showing the mixed responses toward this bacterial gender. Most publications did not report on additional bacterial activity, in accordance with our own findings. As observed in vitro (Van den Abbeele et al. 2013), Lactobacillus modulation, when observed, remained significant after a period of wash-out (Matteuzzi et al. 2004). A reduction of proteolytic activity is consistent with our findings (Lecerf et al. 2012). This was observed by a reduction in phenol (Damen et al. 2012), cresol (Cloetens et al. 2010; Damen et al. 2012; Windey et al. 2014) or branched chain fatty acids (François et al. 2014; Walton et al. 2012). Eventhough pH drop was not measured in the different studies, this may be a consequence in luminal environment unfavourable to proteolytic fermentation following the acid bacteria stimulation (Bifidobacterium, Lactobacillus). Finally, consumption of 2.2g/day of a MC-AX (avDP=18) over 3 weeks increased butyrate production as compared to LC-AX control group (avDP>170). Butyrate stimulation was observed by some authors (Damen et al. 2012; Walton et al. 2012), in agreement with our own data, but not by others (Childs et al. 2014; François et al. 2014; Windey et al. 2014), while another one observed a reduction in butyrate (Maki et al. 2012). Other metabolites were not affected in any of the reports. A number of publications can be found on the prebiotic activity of arabinoxylans or their derivatives. They range from short-term in vitro batch culture to in vitro intestinal simulation models, in vivo animal models and clinical studies. Our clinical study is the only of the 4 models where all markers have been measured (Figure 5). MC-AX stimulates lactic acid bacteria, including Bifidobacterium. By extension, other bacterial families are stimulated and increase production of butyrate and lower the pH. This in turn inhibits proteolytic bacteria (Bacteroides, Clostridium) and their metabolites (p-cresol). These observations are mostly consistent with the literature and with our SHIME® model and the 2 preclinical models using the same product. This is the first time to our knowledge that the Comparison with some other prebiotic molecules is summarized in table 3. At an equivalent dose of 5g/day, only FOS showed an equivalent bifidogenic activity (Bouhnik et al. 2004; Mitsou et al. 2010). Galactooligosaccharides (GOS) on the other hand could have moderate stimulation (Bouhnik et al. 2004; Davis et al. 2010; Mitsou et al. 2010) or strong stimulation of Bifidobacterium (Depeint et al. 2008) or total lactic bacteria (Alander et al. 2001).

5. Conclusions same product has been tested and compared on such a range of complementary models.

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Figures and tables Figure 1: Summary of the four prebiotic protocols Study design, duration and dose (expressed as g per day equivalent of pure MC-AX) of the test product in the four models are represented. Dotted lines represent run-in and non-interventional periods, while full and hashed arrows represent test and placebo treatment periods, respectively.

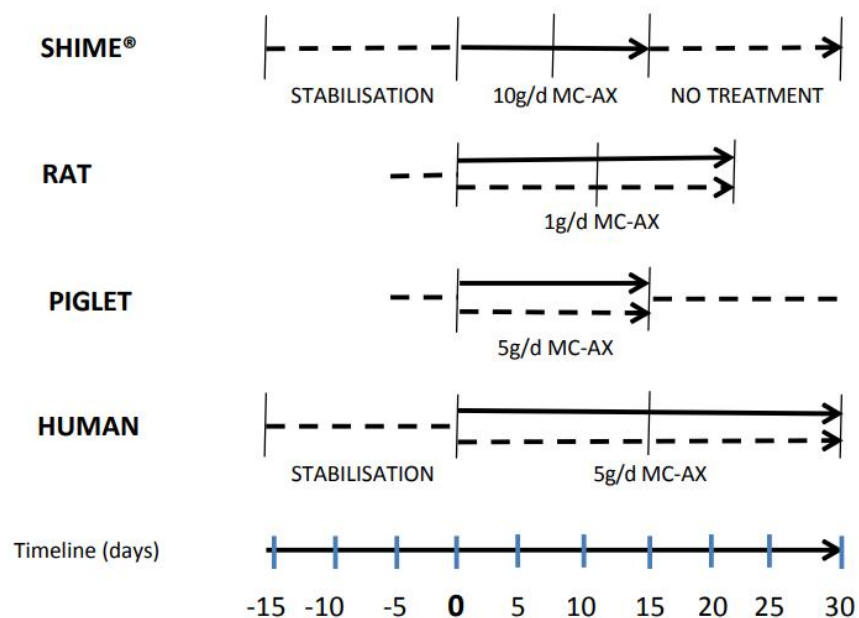




Figure 2: Bifidogenic activity of MC-AX Bifidobacterium was semi-quantified by culture (SHIME®) or qPCR (the other 3 models) and expressed as log unit averages. Data for the SHIME® represent the average value (and standard deviation) of the 3 colonic fermenters. Data for the rat, human and piglet models are expressed as mean and SEM. Models were fed MC-AX treatment (black line) or placebo treatment (dotted line) according to the protocols described in the text. \* and § represent statistical variation from baseline and placebo group, respectively ( $p < 0.05$ ).

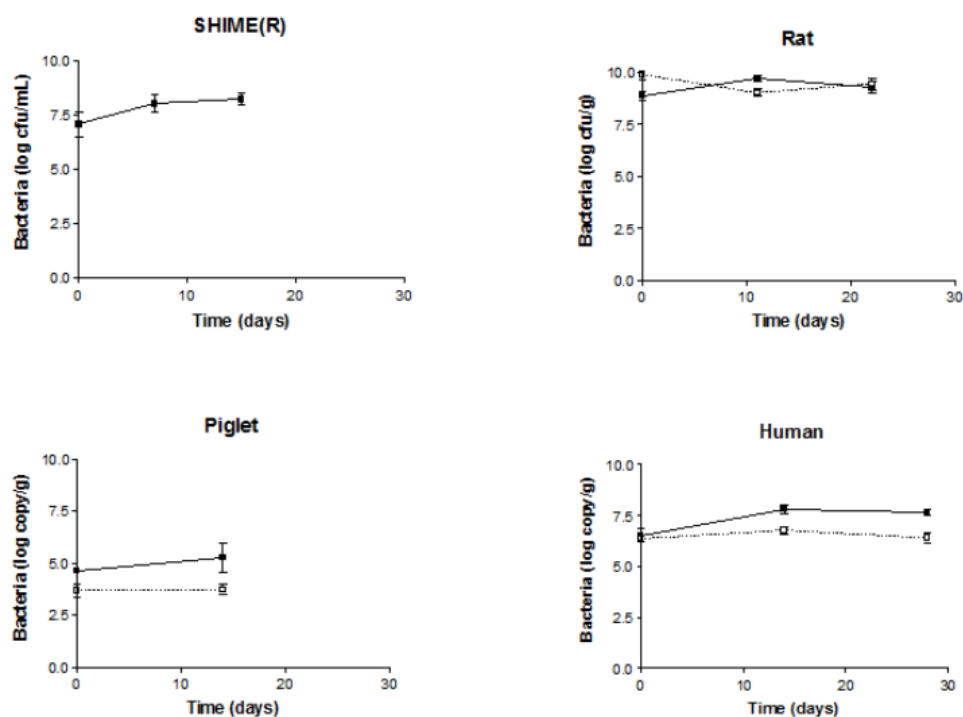


Figure 3: Lactic bacteria stimulation of MC-AX Lactobacillus was semi-quantified by culture (SHIME®) or qPCR (the other 3 models) and expressed as log unit averages. Data for the SHIME® represent the average value (and standard deviation) of the 3 colonic fermenters. Data for the rat, human and piglet models are expressed as mean and SEM. Models were fed MC-AX treatment (black line) or placebo treatment (dotted line) according to the protocols described in the text. \* and § represent statistical variation from baseline and placebo group, respectively ( $p < 0.05$ ).

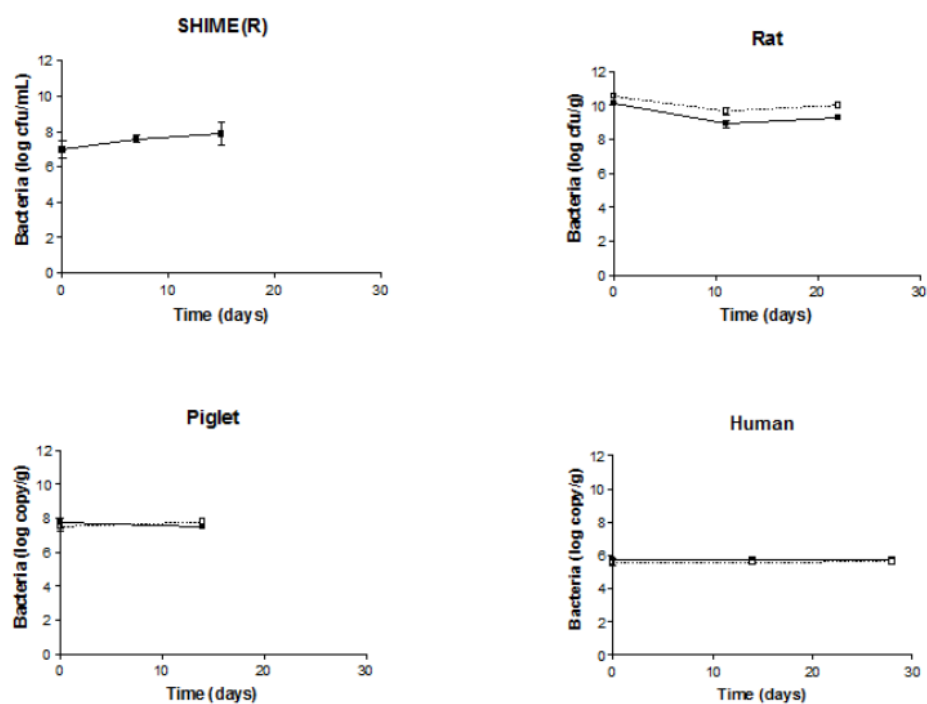


Figure 4: Lactate and short chain fatty acid modulation of MC-AX Lactate was quantified in vitro and short chain fatty acids (SCFA) were quantified in the rat and human studies. Data for the SHIME® represent the average value (and standard deviation) of the 3 colonic fermenters. Data for the rat and human models are expressed as mean and SEM. Models were fed MC-AX treatment (dark grey) or placebo treatment (light grey) according to the protocols described in the text. \* and § represent statistical variation from baseline and placebo group, respectively ( $p < 0.05$ ).

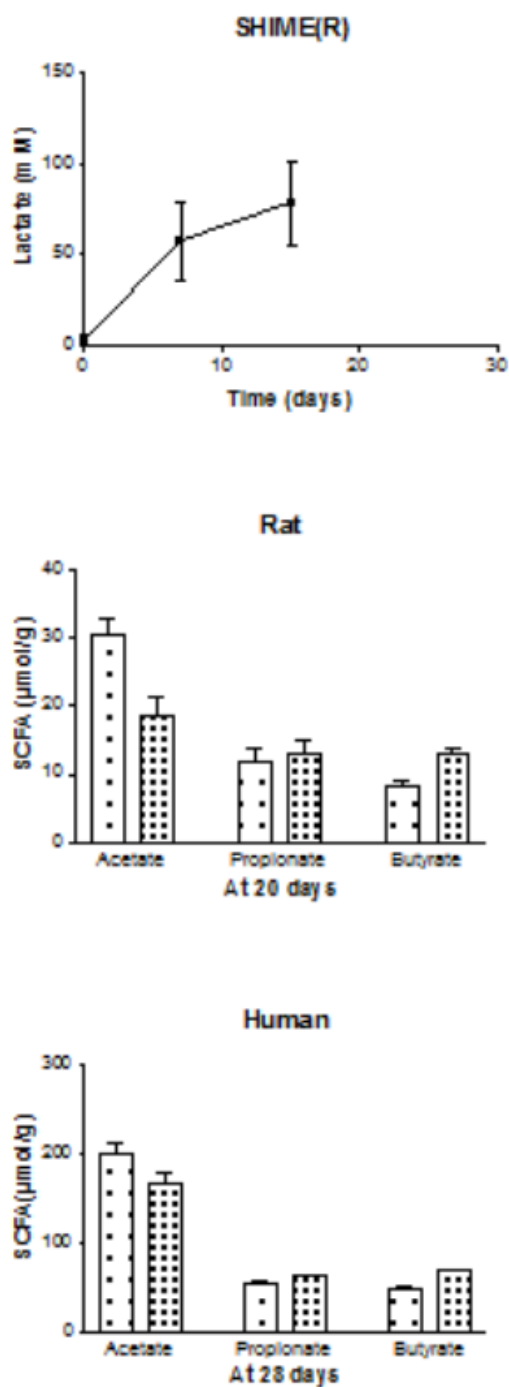
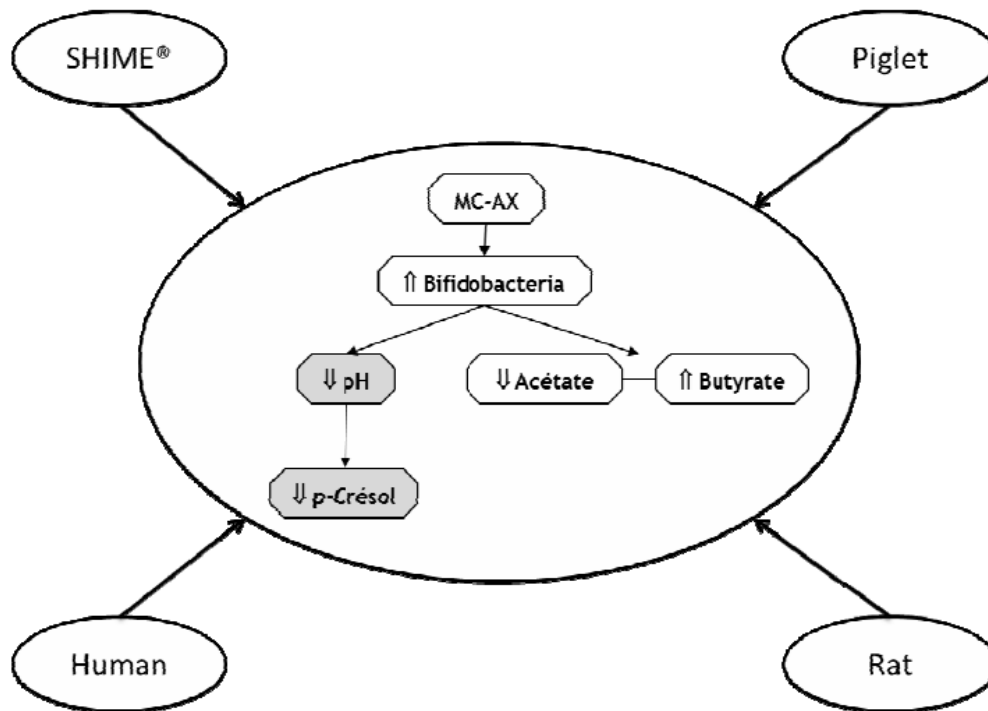


Figure 5- Bifidogenic activity of MC-AX. Diagram of bifidogenic activity and its consequences in terms of intestinal environment. The different relations are validated by at least 2 of the study models presented herein. In grey are links that have been shown by our clinical trial but not measured in the other models.



**Table 1:** Media used for enumeration of microbial groups

Microbial group	Medium
All aerobes	
	Blood Columbia agar
All anaerobes	
Entebacteria	Bromo-Crésol-Purple
Enterococci	Bile-Esculin-Azid
Lactobacillus spp	Man-Rogosa-Sharpe
Bacteroides spp	Bile-Bacteroides-Esculin
Bifidobacterium	Beerens Wilkins-Chalgren agar [70,71]

**Table 2:** In vitro observations on arabinoxylans

Reference	Model	Product	Dose / duration	Bacteria	Metabolites
This study	SHIME®	MC-AX avDP=25; DAS=0.75	10g/L 14 days	<i>Bifidobacterium</i> ++ <i>Lactobacillus</i> ++ <i>Enterobacterium</i> -	Lactate +
Grootaert 2007	Twin-SHIME®	AX avDP=15; DAS=0.27	3g/L 21 days	<i>Bifidobacterium</i> NS <i>Lactobacillus</i> - <i>Roseburia</i> -	Lactate + Butyrate + Acetate -
Van den Abbeele 2013	TIM2	LC-AX avDP>60; DAS=0.7	5g/d 48 hours	<i>Bifidobacterium</i> + <i>Lactobacillus</i> NS	Propionate + Acetate - Butyrate -
	Twin-SHIME®	LC-AX avDP=60; DAS=0.7	3g/L 21 days	<i>Bifidobacterium</i> ++ <i>Lactobacillus</i> ++ <i>Enterococcus</i> -	Propionate + Acetate -
Sanchez 2009	SHIME®	AXOS avDP=29; DAS=0.30	3g/L 21 days	<i>Bifidobacterium</i> + <i>Lactobacillus</i> +	Butyrate + Propionate +/- Acetate +/-
Makelainen 2010	Semi-cont	Xylan DP=35-40	0.5g/d 48 hours	<i>Bifidobacterium</i> ++ <i>Lactobacillus</i> -	Acetate + Butyrate +
Vardakou 2007	MCS	xylanase-WU-AX (F+) avDP=3; DAS=0.69	3g/d 72 hours	<i>Bifidobacterium</i> ++ <i>Bacteroides/Clostridium</i> -	

**Table 3:** Bifidogenic activity across clinical studies

Reference	Product	Dose / duration	<i>Bifidobacterium</i>
Lecerf et al 2012	MC-AX; avDP=25; DAS=0.75	5g/d; 28 days	+ 1.1 log; (data @D14)
Damen et al 2012	In situ MC-AX; avDP=18	2.2g/d; 21 days	NS
Walton et al 2012	In situ MC-AX; aDP=18	2g/d; 14 days	+ 0.26 log
Cloetens et al 2010	SC-AX; avDP=6; DAS=0.26	10g/d; 14 days	+ 0.61 log
Matteuzzi et al 2004	10% AX + 10% Raffinose	10g/d; 21 days	+ 0.17 log
Mitsou et al 2009	SC-FOS	5g/d; 14 days	+ 0.97 log
Bouhnik et al 2004	FOS	5g/d; 14 days	+ 1.38 log
Davis et al 2010	GOS	5g/d; 14 days	+ 0.28 log
Mitsou et al 2010	GOS	0.75g/d; 14 days	+ 0.31 log
Bouhnik et al 2004	GOS	5g /d; 14 days	+ 0.38 log
Depeint et al 2008	GOS	7.5g/d; 7 days	+ 0.98 log