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Assessment of prebiotic potential of Akpan-yoghurt-like product and effects on the human intestinal microbiota

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ABSTRACT

The effect of Akpan (yoghurt-like product) consumption on the human intestinal microbiota was examined. The prebiotic potential of Akpan was assessed by *in vitro* fermentation using, as inocula, individual probiotic strains and faecal samples from three healthy donors. In this latter case, the prebiotic activity was assessed through the quantification of short chain fatty acid (SCFA) production, the evolution of the pH and the evaluation of dynamic bacterial population by fluorescent *in situ* hybridization (FISH). Results demonstrated that Akpan was metabolized after 44 h of fermentation, exerting a prebiotic effect, similar to that observed for fructooligosaccharides (FOS). All the considered bacterial groups significantly increased after Akpan and FOS fermentation. Under the tested conditions, no significant differences between the SCFA concentration produced by Akpan and FOS after of incubation were detected. Our findings contribute to support the utilization of Akpan as a functional food with potential beneficial effects on gastrointestinal health.

1. Introduction

Fermented cereal foods are widely consumed worldwide, especially in many African countries, where they are valued due to their taste, aroma, bioactive constituents, and texture (Franz et al., 2014; Sacca et al., 2012). These foods generally contain health-promoting compounds beyond traditional nutrients as they are considered to have some health benefits, and to aid in the control of some diseases, in particular intestinal

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disorders (Franz et al., 2014; Mathara, Schillinger, Kutima, Mbugua, & Holzapfel, 2004).

Ogi is a starchy product obtained by fermentation of a suspension of wet-milled cereal (commonly maize, although sorghum or millet is also employed as raw material) (Akissoé et al., 2014; Nago, Hounhouigan, Akissoé, Zanou, & Mestres, 1998). It is considered the most important weaning food for infants in West Africa, although it is also consumed by adults (Blandino, Al-Aseeri, Pandiella, Canterob, & Webb, 2003). *Ogi* is used for Akpan preparation, a traditional yoghurt-like product made from fermented cereal starch, and consumed as a thirst-quenching beverage in Benin (Akissoé et al., 2014). Like many other indigenous fermented cereal products, Akpan is appreciated for its sweet but slightly sour taste and the aroma of its active components, which are developed naturally during processing or are added during consumption (Akissoé et al., 2014; Sacca et al., 2012). Akpan is of great economic significance in urban and rural areas in Benin and it will take on increasing importance in the future due to the many variants currently available. Indeed, the consumer demand for non-dairy products such as Akpan (commonly known as "vegetal yoghurt") is a challenge for the food industry in the future (Sacca et al., 2012).

Research studies on Akpan are very limited and focused towards its basic description, as well as to its sensory quality and nutritional attributes (Akissoé et al., 2014). However, to the best of our knowledge, other properties such as the impact of Akpan on human intestinal microbiota have not yet been investigated. Therefore, taking into account that the diet is considered a major driver for changes in gut bacterial diversity that can affect its functional relationships with the host, it seems interesting to assess how this product can affect the intestinal microbial ecosystem (Laparra & Sanz, 2010; Maccaferri et al., 2012). In this context, Maccaferri et al. (2012) demonstrated the ability of different flours (whole grain rye, whole grain wheat, chickpeas and lentils 50:50, and barley milled grains) to modulate the intestinal microbiota composition.

The aim of this work was to evaluate the impact of Akpan on gut microbiota using small-scale *in vitro* batch culture. The prebiotic potential of this product was assessed by measuring the dynamics of selected microbial populations using the fluorescent *in situ* hybridization (FISH) technique. The production of short-chain fatty acids (SCFAs) and the pH evolution were also determined in this work. The assessment of Akpan as fermentable substrate for probiotic bacteria and its influence on their growth and metabolic activity were also studied. The final objective of this study was to contribute to the development of novel marketable functional foods, which can be included in dietary strategies for human health promotion.

2. Materials and methods

2.1. Raw materials and Akpan preparation

Akpan was prepared in the Cirad laboratory using white maize grains provided by Abomey-Calavi University (Cotonou, Benin) as raw material. The traditional process for making Akpan from white maize *ogi* was described by Sacca et al. (2012). It involves the following steps: i) the grains were steeped in boiling water for 5 min and left in water for 24 h until it reached room temperature (grain/water ratio: 1/2), ii) the steeped grains were milled using a grinder (Thermomix Vorwerk; for 1.5 min, depending on the amount) and iii) the wet maize meal was sifted through a 315 μm sieve using a large amount of water (wet flour/water ratio: 1/3) for separating flour and bran. Then, the slurry collected under the sieve was transferred to a crystallizer and a lactic acid starter (*Lactobacillus casei* CNCM I-4592, Lesaffre Company, Marcq-en-Baroeul, France) was added. Finally, the mixture was fermented for 20–24 h in an oven at 35 °C. The starter was previously activated at 35 °C for 5–7 h in a small amount of maize flour slurry obtained as described above. The supernatant of the fermented product was separated. Part of it (85%) was boiled; the other part (15%) was remixed with the fermented flour (*ogi*). The boiled supernatant was added to part of the *ogi* (70%), which was cooked for 15 min at a temperature of 80–90 °C. The 30% of raw *ogi* put aside was added to the cooked *ogi* after cooling and thus, Akpan, a partially cooked product, was obtained. The Akpan was then freeze dried and stored at refrigeration temperature to perform microbiological studies.

2.2. In vitro *digestion of Akpan*

In vitro digestion of Akpan consisted of a simulated gastric digestion, followed by a small intestinal digestion, as described by Mills et al. (2008), with some modifications. Briefly, 8 g of freeze-dried sample was mixed with water (30 mL) and the mixture was homogeneized for 2 min. The solution was then transferred to an Erlenmeyer flask, and the mouth phase was initiated by adding α-amylase (2.6 mg in 2.5 mL of 1 mM $CaCl_2$ solution; pH 7.0) and incubated at 37 °C for 30 min. At the end of the incubation period, the pH value was decreased to 2 by adding 6 M HCl. Then pepsin (0.36 g in 0.625 mL of 0.1 M HCl) was added to the mixture and incubated for 2 h under the same conditions mentioned above. Subsequently, the pH was increased to 7.0 by addition of 6 M NaOH, and pancreatin (0.075 g) and bovine bile salts (0.468 g) dissolved in 3.2 mL of 0.5 M $NaHCO_3$ were added to start the small intestinal phase. The resulting slurry was then transferred to a 1 kDa molecular weight cut-off (MWCO) dialysis tubing and dialysed for 2 d at 20 °C in order to remove low molecular mass digestion products. Finally, the resulting sample was freeze-dried and stored for fermentation experiments.

2.3. *Evaluation of Akpan as a source of fermentable carbohydrates for probiotic bacteria growth*

2.3.1. *Microorganisms and growth conditions*

Four bacterial strains were used in this study: *L. casei* L431 and *Bifidobacterium lactis* B94 (from DELVO PRO LAFTI; DSM, Heerlen, The Netherlands) and *L. casei* L01 and *Bifidobacterium animalis* Bb12 (from Chr. Hansen; Hørsholm, Denmark). Strains were stored at –80 °C in de Man–Rogosa–Sharpe (MRS) broth (Biokar Diagnostics, Beauvais, France) with 30% (v/v) glycerol. Before the assays, all strains were grown in MRS broth at 37 °C for 16 h. Anaerobic conditions were used for *B. lactis* B94 and *B. animalis* Bb12, whereas aerobic atmosphere was used for lactobacilli strains. All tested strains are considered as probiotics as

previously reported in different studies (Hernandez-Hernandez et al., 2012; Rodrigues et al., 2011). Fermentation assays were carried out for each strain in media containing: MRS-medium supplemented with 1% (w/v) of Akpan, 1% (w/v) fructooligosaccharides (FOS, used as reference) and MRS medium without carbon source at 37 °C for 48 h.

2.3.2. In vitro *fermentation assays*

The composition of the de Man, Rogosa, and Sharpe (MRS) basal media (pH 6.8) employed in this study was as follows: 10 g/L of protease peptone, 10 g/L of beef extract, 5 g/L of yeast extract, 1 g/L of Tween 80, 2 g/L of ammonium citrate, 5 g/L of sodium acetate, 0.1 g/L of magnesium sulphate, 0.05 g/L of manganese sulphate, 2 g/L of dipotassium sulphate, 0.05 g/L of cysteine hydrochloride (before inoculation in the case of anaerobic strain), and 1% (w/v) of Akpan or FOS. FOS was used as positive control due to its well-established prebiotic properties, whereas MRS without any carbon source was used as negative control. The media were sterilized at 110 °C for 20 min, and the incubation was carried out at 37 °C using an inoculum made up of *Lactobacillus* or *Bifidobacterium* cells (grown on MRS broth media for 16 h). All fermentations were started with ca. 10^6 CFU/mL. Samples of the fermentation broths were withdrawn at 0, 5, 10, 24 and 48 h for metabolite analysis and cell counting. Viable cells were quantified by plating on MRS agar (supplemented with cysteine hydrochloride in the case of *Bifidobacterium*) and incubation at 37 °C for 48 h (anaerobic and aerobic conditions were used for *Bifidobacterium* and *Lactobacilli* strains, respectively). All experiments were carried out in duplicate. Metabolite production was determined by HPLC using the method described in section 2.4.4.

2.4. *Prebiotic effect*

2.4.1. *Faecal samples and* in vitro *fermentations*

Faecal samples were obtained from healthy human donors (three females; age 25–35 years old), who ingested a normal diet, without any known metabolic or gastrointestinal disorders and had not taken antibiotics 3 months prior to faecal sample donation. Faeces were collected and kept in an anaerobic cabinet (Model Bactron IV, Shel-Lab, Cornelius, OR, USA) for a maximun of 2 h after collection. The faecal inocula (FI) were prepared as per Gullón et al. (2014).

Samples of Akpan were assayed for their *in vitro* fermentability according the method of Gullón et al. (2014). Fermentations were run over a period of 44 h, and 3 mL samples were taken at 0, 6, 24 and 44 h to be analysed for their bacterial populations by fluorescent *in situ* hybridization (FISH). The pH of the medium was also measured and lactic acid and short chain fatty acids (SCFAs) were quantified by HPLC. All additions and inoculations were carried out inside an anaerobic cabinet (5% H_2, 10% CO_2 and 85% N_2).

2.4.2. *Bacterial enumeration by fluorescent* in situ *hybridization (FISH) assays*

FISH was performed as previously described (Gullón, Gullón, Sanz, Alonso, & Parajó, 2011). Synthetic oligonucleotide probes (TIB MOLBIOL, S.L., Spain) targeting specific regions of the 16S rRNA molecule and labelled with the fluorescent dye Cy3 (Sigma) were utilized for the enumeration of bacterial groups: Bif164, specific for the *Bifidobacterium* genus; Lab158, for the *Lactobacillus–Enterococcus* group; Bac303, specific for the *Bacteroides–Prevotella* group; His150, for the *Clostridium histolyticum* subgroup; Erec482 for the *Ruminococcus–Eubacterium–Clostridium* cluster and Fpra655 for *Faecalibacterium prausnitzii* and relatives. Total cell counts were performed by staining with 4′,6-diamidino-2-phenylindole (DAPI). Cells were counted using an epifluorescence microscope (Olympus BX41). A minimum of 10 fields were counted per sample analysed.

2.4.3. *Fermentation metabolites analyses*

Supernatants from the fermentation media were filtered through 0.20 μm cellulose acetate membranes. Aliquots of the filtered samples were assayed for organic acids (succinic, lactic, formic, acetic, propionic and butyric acids) using an Agilent 1200 series HPLC instrument with a refractive index (RI) detector (Agilent, Waldbronn, Germany) operated at 50 °C. Other analysis conditions were as follows: Aminex HPX-87H column (from BioRad, Hercules, CA, USA); mobile phase, 0.003 M H_2SO_4; flow, 0.6 mL/min.

2.5. *Statistical analysis*

Statistical analysis was performed using SPSS for Windows version 21.0 (IBM SPSS, Chicago, IL). Univariate analysis of variance (ANOVA) and the post hoc Tukey test were used to determine the significance of the effect of Akpan on bacterial group populations and SCFA production. Differences were considered significant at the 5% level.

3. Results and discussion

3.1. *Effects of Akpan on the probiotic growth*

In order to assess the suitability of Akpan as a fermentable carbohydrate source for probiotic bacteria, *in vitro* fermentation assays were carried out using two *Lactobacillus* strains (*L. casei* L431 and *L. casei* L01) and two *Bifidobacterium* strains (*B. animalis* Bb12 and *B. lactis* B94). Fig. 1 shows the time course of the number of viable cells (expressed as log CFU/mL) for the different strains tested as well as pH evolution in the fermentation media. As expected, a very limited growth was observed in the negative control assay confirming that the growth enhancement was a consequence of the carbohydrates added (Akpan and FOS).

Regarding the *Bifidobacteria* strains, a stimulatory effect on the growth was observed after 48 h using both Akpan and FOS. The viable cell numbers of *B. animalis* reached a value of 8.07 log CFU/mL (as average) for both fermentable carbohydrate sources. However, some differences were observed in the case of *B. lactis* B94; the number of viable cells at the end of fermentation was slightly higher in media containing FOS (8.75 log CFU/mL and 8.24 log CFU/mL for FOS and Akpan, respectively). The pH showed a similar trend for both carbohydrate sources, when the medium was inoculated with *B. animalis* Bb12 (Fig. 1). By contrast, when *B. lactis* B94 was cultured in media containing FOS, a higher reduction in pH was observed in

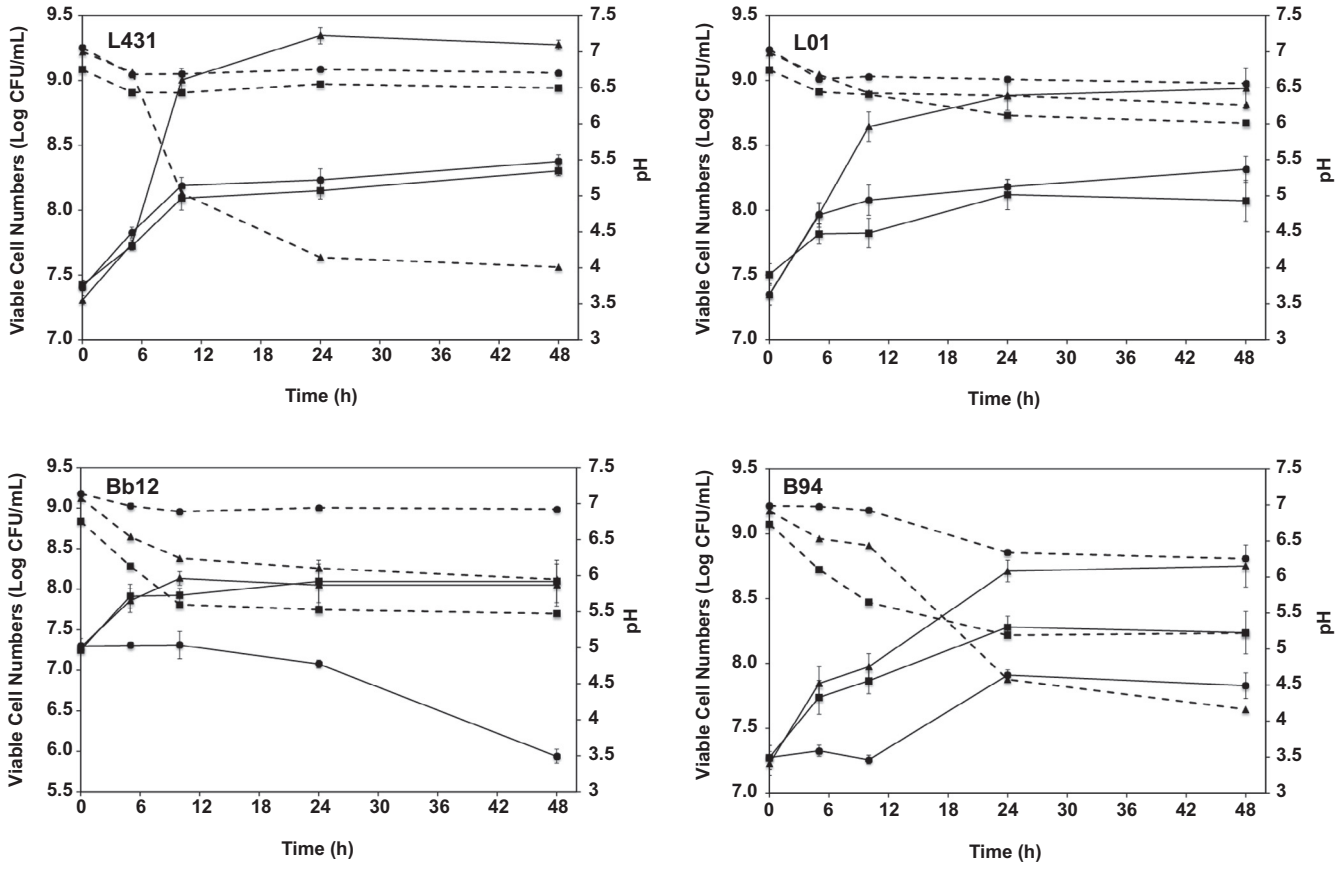

Fig. 1 – Viable cell numbers (log CFU/mL) (___) of *Lactobacillus casei* L431, *Lactobacillus casei* L01, *Bifidobacterium animalis* Bb12 and *Bifidobacterium lactis* B94 and pH (—) values throughout incubation time (48 h) at 37 °C growing in MRS-medium supplemented with 1% (w/v) of Akpan (■), 1% (w/v) FOS (▲) and MRS medium without fermentable carbohydrates (●). Values are expressed as the mean ± standard deviation (SD) of independent duplicate assays.

comparison with Akpan (final pH = 4.2 and 5.2 for FOS and Akpan, respectively). These results are in agreement with those obtained for the viable cell numbers (*B. lactis* B94 reached higher values in media containing FOS).

Regarding *L. casei* L431 and *L. casei* L01, no growth enhancement was observed for Akpan (Fig. 1), showing similar profiles to the one observed in the negative control. However, the decrease in pH values was similar for both Akpan and FOS, containing cells of *L. casei* L01. After 48 h of fermentation, pH values of 6 and 6.3 were reached in media containing Akpan and FOS, respectively. In the case of *L. casei* L431, the pH decreased to 4 for media containing FOS after 48 h. When this strain was cultured in media containing Akpan, the pH remained almost constant.

HPLC analysis confirmed the presence of lactate, formate and acetate as fermentation products in the cell-free culture supernatants. However, variations in the production of these acids for the various strains and substrates were observed (Table 1). In general, strains *L. casei* L431 and *B. lactis* B94 grown in media containing FOS produced higher lactic acid concentrations than those achieved when Akpan is used as substrate. Acetic acid levels were higher in media containing Akpan in comparison with FOS for both *L. casei* L431 and *B. lactis* B94. Cultures of strains *L. casei* L01 and *B. animalis* Bb12 produced similar amounts of lactic and formic acids, regardless of the substrates used as fermentable carbohydrates. However, these strains produced a higher amount of acetic acid in the media containing Akpan.

Recently, Tadayoni, Sheikh-Zeinoddin, and Soleimanian-Zad (2015) observed that *Lactobacillus plantarum* A7 can metabolize polysaccharides from acorn. The effectiveness of a prebiotic carbohydrate depends on its ability to be selectively fermented by specific targeted organisms. This is in agreement with a number of studies that demonstrate that the ability of lactobacilli and bifidobacteria to ferment prebiotic carbohydrates depends on the strain and substrate tested (Huebner, Wehling, & Hutkins, 2007).

Akpan is obtained from fermented cereal, and contains significant amounts of polysaccharides that act as fermentable carbohydrates for supporting the growth of *Lactobacillus* and *Bifidobacterium* strains, resulting in the production of organic acids.

3.2. *Effects of Akpan on the intestinal microbiota*

3.2.1. *Changes in bacterial populations during* in vitro *batch culture fermentation*

In this work, *in vitro* modification of the faecal microbiota by Akpan and FOS was monitored by FISH. The data shown in Table 2 demonstrate that the total cell counts (DAPI)

Table 1 – Organic acids concentrations (mM) along the fermentation using Akpan or FOS as substrates in media inoculated with *Lactobacillus casei* (L431), *Lactobacillus casei* (L01), *Bifidobacterium animalis* (Bb12) or *Bifidobacterium lactis* (B94).

Fermentable carbohydrate	Acid	Time (h)	L431	L01	Bb12	B94
Akpan	Lactic	5	4.67 ± 0.15	4.86 ± 0.42	2.32 ± 0.21	1.15 ± 0.27
		10	11.26 ± 0.24	5.88 ± 0.89	2.17 ± 0.74	1.24 ± 0.34
		24	7.96 ± 1.78	6.58 ± 1.25	0.97 ± 0.12	9.09 ± 1.21
		48	9.07 ± 2.31	6.89 ± 1.33	0.45 ± 0.24	9.45 ± 1.56
	Formic	5	2.26 ± 0.45	1.72 ± 0.48	1.16 ± 0.21	0.00 ± 0.00
		10	5.45 ± 1.23	0.00 ± 0.00	6.71 ± 1.55	3.28 ± 1.21
		24	5.19 ± 0.75	0.00 ± 0.00	7.42 ± 0.33	7.02 ± 0.75
		48	0.00 ± 0.00	0.00 ± 0.00	3.95 ± 1.80	0.00 ± 0.00
	Acetic	5	6.30 ± 1.25	5.90 ± 1.34	7.45 ± 2.34	9.97 ± 1.27
		10	16.23 ± 3.24	12.81 ± 3.12	13.47 ± 3.47	13.34 ± 2.87
		24	18.72 ± 4.21	23.78 ± 5.25	11.86 ± 2.41	17.79 ± 3.24
		48	19.81 ± 1.34	24.43 ± 4.85	13.23 ± 1.29	19.24 ± 3.78
FOS	Lactic	5	5.90 ± 1.27	6.39 ± 0.78	2.22 ± 0.37	16.62 ± 2.24
		10	59.09 ± 12.45	7.32 ± 0.36	0.00 ± 0.00	31.52 ± 4.34
		24	110.42 ± 14.45	7.05 ± 1.21	0.00 ± 0.00	47.97 ± 8.12
		48	109.13 ± 7.25	9.54 ± 1.78	0.00 ± 0.00	92.50 ± 7.45
	Formic	5	1.59 ± 0.23	1.05 ± 0.10	4.66 ± 10.35	3.39 ± 0.21
		10	0.00 ± 0.00	5.93 ± 1.00	7.30 ± 0.78	7.56 ± 1.27
		24	0.00 ± 0.00	2.13 ± 0.17	7.09 ± 1.98	0.00 ± 0.00
		48	0.00 ± 0.00	0.00 ± 0.00	5.88 ± 0.74	0.00 ± 0.00
	Acetic	5	3.69 ± 1.25	4.44 ± 0.38	5.34 ± 1.00	9.56 ± 2.48
		10	5.00 ± 1.64	9.29 ± 1.25	5.44 ± 0.87	10.91 ± 1.39
		24	3.79 ± 0.87	15.05 ± 2.38	5.53 ± 1.21	13.22 ± 0.15
		48	9.31 ± 1.21	19.03 ± 3.45	6.99 ± 1.11	17.10 ± 1.72
	Lactic	5	5.26 ± 0.64	3.80 ± 0.11	0.00 ± 0.00	0.30 ± 0.02
		10	5.21 ± 0.13	3.53 ± 0.10	0.00 ± 0.00	0.97 ± 0.01
		24	4.11 ± 0.42	2.55 ± 0.16	0.00 ± 0.00	1.41 ± 0.14
		48	4.66 ± 0.23	2.88 ± 0.17	0.00 ± 0.00	1.14 ± 0.29
	Formic	5	5.15 ± 0.31	2.26 ± 0.29	0.69 ± 0.11	2.64 ± 0.37
		10	3.91 ± 0.10	7.57 ± 0.42	7.56 ± 0.79	6.83 ± 1.15
		24	0.00 ± 0.00	3.56 ± 0.32	4.53 ± 0.25	5.47 ± 0.55
		48	0.00 ± 0.00	0.00 ± 0.00	6.11 ± 1.19	5.67 ± 0.39
	Acetic	5	5.25 ± 0.91	7.28 ± 0.55	0.00 ± 0.00	0.00 ± 0.00
		10	6.90 ± 0.74	9.16 ± 0.98	2.50 ± 0.12	4.25 ± 0.21
		24	8.99 ± 0.39	9.16 ± 1.12	2.33 ± 0.09	5.33 ± 0.33
		48	9.66 ± 1.12	6.83 ± 0.33	5.38 ± 0.28	0.00 ± 0.00

Values are expressed as mean ± standard deviation (SD) of two replicates.

increased significantly with time in experiments containing FOS and Akpan. On the contrary, in negative control cultures, no variations were observed in the bacterial growth. In media containing FOS and Akpan, the total cell counts increased (as average) up to 17% at 44 h (this value did not show significant differences ($p > 0.05$) for both Akpan and FOS).

Regarding *Bifidobacterium* population, the data confirmed a preferential growth of this genus when FOS or Akpan was used

Table 2 – Changes in bacteria population (expressed as log cells/mL) after 0, 6, 24 and 44 h *in vitro* fermentation of FOS, Akpan and negative control.

Group	FOS				Akpan			Negative control		
	0 h	6 h	24	44 h	6 h	24	44 h	6 h	24	44 h
DAPI	8.13 ± 0.04	9.04^{b} ± 0.36	9.87^{b} ± 0.23	9.66^{b} ± 0.31	9.13^{b} ± 0.05	9.61^{b} ± 0.05	9.89^{b} ± 0.21	8.50^{a} ± 0.03	8.52^{a} ± 0.12	8.37^{a} ± 0.02
Bif164	7.46 ± 0.11	8.43^{b} ± 0.34	8.67^{b} ± 0.04	8.65^{b} ± 0.14	8.45^{b} ± 0.05	8.89^{c} ± 0.06	8.86^{b} ± 0.06	7.90^{a} ± 0.05	7.71^{a} ± 0.06	7.64^{a} ± 0.14
Lab158	7.01 ± 0.10	8.66^{b} ± 0.17	8.58^{b} ± 0.16	8.47^{b} ± 0.12	8.51^{b} ± 0.04	8.93^{c} ± 0.05	8.96^{c} ± 0.15	7.87^{a} ± 0.11	7.69^{a} ± 0.06	7.40^{a} ± 0.13
Chis150	7.49 ± 0.13	8.89^{c} ± 0.14	8.83^{b} ± 0.10	8.81^{b} ± 0.23	8.64^{b} ± 0.03	9.00^{b} ± 0.08	8.91^{b} ± 0.15	8.10^{a} ± 0.05	7.92^{a} ± 0.05	7.83^{a} ± 0.12
Bac303	7.88 ± 0.01	8.83^{b} ± 0.11	8.96^{b} ± 0.08	8.93^{b} ± 0.18	8.71^{b} ± 0.07	8.93^{b} ± 0.02	8.83^{b} ± 0.09	8.08^{a} ± 0.11	7.90^{a} ± 0.13	7.84^{a} ± 0.20
Erec482	7.51 ± 0.03	8.78^{b} ± 0.21	9.00^{c} ± 0.07	8.84^{b} ± 0.20	8.46^{b} ± 0.09	8.82^{b} ± 0.08	8.76^{b} ± 0.08	8.04^{a} ± 0.05	7.74^{a} ± 0.05	7.68^{a} ± 0.06
Frap655	7.50 ± 0.14	8.60^{b} ± 0.23	8.78^{b} ± 0.04	8.62^{b} ± 0.07	8.81^{b} ± 0.08	9.00^{c} ± 0.04	8.87^{c} ± 0.07	7.92^{a} ± 0.06	7.99^{a} ± 0.07	7.86^{a} ± 0.05

Total bacterial counts were obtained using DAPI and bacterial groups with FISH probes. Mean values ± SD (n = 3). Different letters indicate significant differences ($P \leq 0.05$) for each bacterial group. Substrates were compared at three fermentation sampling times (6, 24 and 44 h).

as substrate in comparison with the negative control. Significant growth ($p \leq 0.05$) was observed in the number of bifidobacteria at 24 h when Akpan was employed. After 44 h, this number increased by 14 and 17% in experiments containing FOS and Akpan, respectively. FOS has previously been reported to enhance the growth of bifidobacteria (Rivas, Gullón, Gullón, Alonso, & Parajó, 2012; Rossi et al., 2005), but the bifidogenic activity of Akpan is new in the scientific literature and these results show the bifidogenic effect of this functional beverage. The results obtained in this work are in agreement with the data published with similar substrates. Costabile et al. (2008) reported an increased number of faecal bifidobacteria after ingestion of the wholegrain wheat breakfast cereal. Similarly, an increase in bifidobacteria levels was also found during the fermentation of arabinoxylooligosaccharides from wheat bran (Gullón et al., 2014).

The *Lactobacillus–Enterococcus* group population was also quantified in this work. A higher increase during incubation with FOS and Akpan was observed with respect to the control negative. In experiments containing Akpan, the levels of *Lactobacillus–Enterococcus* increased significantly after 24 and 44 h of incubation in comparison with the results obtained with FOS ($p \leq 0.05$). At 24 h of fermentation, the *Lactobacillus* counts increased by 17 and 22% in cultures containing FOS and Akpan, respectively. The differences observed in the fermentation of Akpan using faecal inoculum were not in agreement with the results obtained in pure cultures with *Lactobacillus* cells where a low growth was observed. This fact can be explained on the basis of a variety of different species that are present in faecal inocula, enabling the complete fermentation of carbohydrates (Pastell, Westermann, Meyer, Tuomainen, & Tenkanen, 2009). In many cases, the metabolic end products generated by some species can be used as substrates by other microorganisms that cannot metabolize the original substrate (Gibson & Roberfroid, 1995). This is the first evidence that shows the stimulatory effect on intestinal lactobacilli populations with Akpan.

Regarding *C. histolyticum* group Clusters I and II, the data show that growth of this group was statistically significant in cultures with FOS during the first 6 h of fermentation (see Table 2). On the other hand, after 24 and 44 h of incubation, no significant differences were observed between FOS and Akpan. In a related study, Salazar et al. (2009) reported an increase of this type of bacteria after 10 h using inulin as fermentable carbohydrate, whereas Sarbini et al. (2011) did not observe a significant increase of these clusters when fermented dextrans were used. From the point of view of the health effects, clostridia are mainly considered as harmful bacteria.

Bacteroides–Prevotella is one of the predominant bacterial group in gut microbiota, and it is a metabolically versatile group as it can use many types of polysaccharides and produce acetic acid and propionic acid (Salazar et al., 2009). As expected, FOS and Akpan promoted the growth of *Bacteroides–Prevotella* in faecal cultures, although no significant differences were observed among both fermentable carbohydrates (see Table 2).

The *Clostridium coccoides–Eubacterium rectale* group and *F. prausnitzii* play a physiological role related to their ability to produce relatively large amounts of butyrate, which has been shown to exert beneficial effects on gut health (Scott, Martin, Duncan, & Flint, 2014). Regarding *C. coccoides–E. rectale*, gradual increases in counts were observed during fermentations in cultures with FOS and Akpan. After 44 h of fermentation, the numbers increased (by an average) by 15% in cultures containing FOS or Akpan. For instance, Kleessen, Hartmann, and Blaut (2001) reported increased levels of *C. coccoides–E. rectale* in a human-flora-associated (HFA) animal study after ingestion of inulin.

A significant increase in *F. prausnitzii* was also detected for both substrates, although these changes are slightly more noticeable for Akpan. After 24 h, the number of bacteria belonging to this group increased (by an average) by 14%. Similar results were observed using commercial prebiotics. For instance, Dewulf et al. (2013) demonstrated that the dietary supplementation with a mixed scFOS/inulin prebiotic stimulated numbers of *F. prausnitzii*. Davis, Martínez, Walter, Goin, and Hutkins (2011) also observed an increase in the number of *F. prausnitzii* after galactooligosaccharides intake. The fact that Akpan stimulated the growth of *F. prausnitzii* is important because this group can be used as an indicator of intestinal health. In this context, the modulation of *F. prausnitzii* abundance (for example, by using functional ingredients such as prebiotics and/or probiotics) might have therapeutic applications in human health (Miquel et al., 2013).

3.2.2. *Changes in lactic acid and short chain fatty acid profile in faecal cultures*

Table 3 summarizes the changes in SCFA and lactate concentrations along the fermentation of media containing Akpan or FOS, as well as the pH variations. The samples were analysed at 0, 6, 24 and 44 h. As can be expected, the increase in total SCFAs and lactate was higher in the media containing Akpan or FOS than in faecal cultures lacking fermentable carbohydrates.

As can be seen, both Akpan as FOS were metabolized, leading to a reduction of the pH values. These decreases were more pronounced than the ones observed in control cultures, in which the pH variation was mainly ascribed to the SCFA generation from protein by putrefactive bacteria present in the intestinal microbiota (Conlon & Bird, 2015; Hartemink, Schoustra, & Rombouts, 1999). The highest concentrations in total SCFA were detected after 44 h of fermentation for media containing FOS and Akpan, although no significant differences were found between both fermentable carbohydrates (see Table 3).

Succinic and formic acid (two intermediary products mainly produced by bifidobacteria) were also quantified. Scarce production of succinic acid was observed at short fermentation times (data not shown). Regarding the formic acid, a substantial increase was observed during the period 0–24 h, although no significant differences were detected between FOS and Akpan. The highest concentrations for formic acid (23.59 mM from FOS and 20.27 mM from Akpan) were achieved after 24 h.

On the other hand, lactic acid (a typical metabolic product of bifidobacteria and lactic bacteria) was also determined, being more abundant in the experiments containing FOS. No lactic acid was observed after 24 h of fermentation in any assay, because it can be utilized by other intestinal bacteria as a carbon source for producing acetate, propionate, and/or butyrate, suggesting a cross-feeding mechanism (Belenguer et al., 2006). As

Table 3 – Concentration (mM) of SCFA (acetate, propionate and butyrate), lactate and formate, and pH variation during *in vitro* fermentation of Akpan, FOS and negative control at 6, 24, and 44 h.

Fermentable carbohydrate	Time (h)	pH	Lactate	Formate	Acetate	Propionate	Butyrate	Total SCFA
Akpan	0	7.2	0.14 ± 0.07	0.34 ± 0.15	1.28 ± 0.45	0.05 ± 0.00	0.24 ± 0.13	2.05 ± 0.19
	6	6.5	1.03^{b} ± 0.06	4.34^{b} ± 2.45	5.57^{a} ± 1.03	2.93^{b} ± 0.63	0.00^{a} ± 0.00	8.51^{a} ± 0.58
	24	5.8	2.57^{a} ± 0.62	20.27^{b} ± 4.26	32.86^{b} ± 3.47	26.37^{b} ± 4.80	1.75^{a} ± 1.27	60.99^{b} ± 9.51
	44	5	0.00^{a} ± 0.00	16.71^{b} ± 5.16	44.46^{b} ± 3.43	32.13^{b} ± 6.46	5.23^{a} ± 0.31	81.83^{b} ± 10.05
FOS	0	7.2	0.18 ± 0.10	0.45 ± 0.12	2.75 ± 0.89	0.38 ± 0.16	0.45 ± 0.11	4.21 ± 0.25
	6	6.0	2.60^{c} ± 0.68	5.77^{b} ± 0.74	13.34^{b} ± 4.35	3.15^{b} ± 1.05	1.04^{b} ± 0.46	17.50^{b} ± 5.85
	24	5.0	11.07^{b} ± 7.43	23.59^{b} ± 1.87	46.86^{c} ± 2.29	11.35^{a} ± 2.42	13.09^{b} ± 2.77	71.32^{b} ± 2.73
	44	4.5	0.68^{a} ± 0.74	21.21^{b} ± 1.33	47.49^{b} ± 9.12	11.16^{a} ± 1.69	23.78^{b} ± 0.92	82.44^{b} ± 8.13
Negative control	0	7.2	0.11 ± 0.05	0.13 ± 0.08	1.45 ± 0.29	0.14 ± 0.09	0.00 ± 0.00	1.83 ± 0.11
	6	6.9	0.00^{a} ± 0.00	0.00^{a} ± 0.00	3.17^{a} ± 0.31	0.95^{a} ± 0.20	0.096^{a} ± 0.16	4.22^{a} ± 0.50
	24	6.8	0.00^{a} ± 0.00	0.00^{a} ± 0.00	20.38^{a} ± 2.67	4.68^{a} ± 1.83	$3.99^{a,b}$ ± 0.59	29.06^{a} ± 3.50
	44	7.2	0.00^{a} ± 0.00	0.00^{a} ± 0.00	29.20^{a} ± 0.82	6.21^{a} ± 1.23	5.14^{a} ± 0.19	40.55^{a} ± 0.52

Starting concentrations of the test substrates were 1% (w/v). Mean values ± SD (n = 3). Different letters indicate significant differences ($p \leq 0.05$) for the same acid. Substrates were compared at three fermentation sampling times (6, 24 and 44 h).

expected, no lactic acid was detected in the negative control experiment.

Acetate is the main SCFA in the gut, and it was the most abundant SCFA in the fermentation media in this work. FOS fermentation resulted in significantly higher amounts at 24 h compared to Akpan ($p \leq 0.05$). The highest concentrations were achieved after 44 h (47.49 mM and 44.46 mM from FOS and Akpan, respectively). As *Bifidobacterium* are acetic acid producers (Gullón et al., 2014; Schiffrin & Blum, 2002), the increase of acetic acid during fermentation correlated well with the increase in the populations of *Bifidobacterium* for both Akpan and FOS (see Table 2). The predominant formation of acetic acid is in line with the results reported by Connolly, Tuohy, and Lovegrove (2012), when were fermented whole grain oat-based cereals using faecal inocula.

Regarding propionic acid concentration, the highest amounts of this acid were found in the experiments containing Akpan (32.13 mM after 44 h), showing significant differences (ca. 3 times) ($p \leq 0.05$) when compared to FOS at 24 and 44 h. This positive modulation was also found during the fermentation of other carbohydrate sources such as arabinoxylooligosaccharides from wheat bran (Gullón et al., 2014) and arabinoxylans from brewer's spent grain (Reis et al., 2014).

It should be noted that propionate is a gluconeogenerator, which has been shown to inhibit biosynthesis of cholesterol and fatty acids in liver. Therefore, carbohydrates that can decrease the acetate : propionate ratio may reduce serum lipids and possibly cardiovascular disease risk (Delzenne & Kok, 2001; Wong, de Souza, Kendall, Emam, & Jenkins, 2006). In this work, the values for acetic acid/propionic acid ratio at 44 h were 1.38 for Akpan and 4.25 for FOS. The result obtained for Akpan compared favourably with those achieved with other substrates in related studies. Gómez et al. (2014) carried out a similar study using pectin from orange peel wastes resulting in an acetic acid/propionic acid ratio of 3.88 at 48 h. Reis et al. (2014) reported average values of 2.5 at 48 h using arabinoxylans. These results confirm that Akpan could be a promising alternative for reducing serum lipids, since very low acetate/propionate ratios were obtained if they are compared to other potential or commercial prebiotics.

Butyrate has an important role as a carbon source for the colonic mucosa, and it is considered a healthy metabolite, since it positively influences cell growth and differentiation, exerts anti-inflammatory effects and has been linked to a reduced incidence of colon cancer (Laparra & Sanz, 2010; Wong et al., 2006). The butyrate production in media containing FOS was more pronounced than in cultures with Akpan, showing significant differences ($p \leq 0.05$). This result can be related to the higher content of lactic acid during the period of 6–24 h for cultures containing FOS, and, by a cross-feeding mechanism, this acid can be converted into butyrate by common intestinal microbiota (Russell, Hoyles, Flint, & Dumas, 2013). The highest butyrate concentration for FOS was achieved after 44 h (23.78 mM), whereas the media containing Akpan showed a significantly lower concentration (5.23 mM). Similarly, Connolly et al. (2012) also observed moderate increases in butyrate levels in media containing different processed breakfast cereals. Higher amounts of butyrate were also obtained for FOS in comparison with AOS in previously reported studies (Gullón et al., 2014; Reis et al., 2014).

4. Conclusions

This work is the first study that shows the effects of Akpan on the complex faecal human microbiota. The results obtained here demonstrated that Akpan can be considered to exert potential prebiotic effects, since it promotes the growth of beneficial colonic bacterial populations and the production of SCFA. Akpan can stimulate the growth of certain bacterial groups in the human intestinal microbiota in an extent comparable to FOS. Moreover, the fermentation of Akpan and FOS resulted in similar SCFA productions, although the SCFA profile was different; Akpan fermentation resulted in a remarkable generation of propionate, while FOS fermentation led to higher concentrations of butyrate. Therefore, the results of this work could support that the consumption of this cereal-based fermented beverage could improve the gut health.

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