

# *In-Vitro* Metabolite Colonic Production from *Otili* (*Sphenostylis stenocarpa*) as Influenced by Selected Gastrointestinal Microbes

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## Abstract

In human nutrition, dietary fibers are un-degradable by mammalian enzymes, and are therefore potentially available for fermentation by micro-organisms living in the gastrointestinal tract. It is thought that these fermentable carbohydrates affect fermentation by stimulating the growth or metabolism of specific bacterial species, which are potentially beneficial for health. Hence in this study three gut associated microbes—*Escherichia coli*, *Bacillus subtilis*, and *Lactobacillus acidophilus* were made to ferment the non-digestible fraction of *Otili*—*Sphenostylis stenocarpa*, a well-established underutilized wild bean with high economic importance. After 18 hours' fermentation period short chain fatty acids (SCFAs) and medium chain fatty acids (MCFAs) were detected and evaluated by GC-MS analysis. Metabolic products were relatively dependent on the fermenter. This present study affirmed butyric acid as the main SCFAs after 18 hours' fermentation. The clinical significance of thirteen other MCFAs detected and quantified was also explored thus conferring a valuable prebiotic on *Otili*.

## Keywords

Indigestible Fraction, Dietary Fibres, Fermentation, Short Chain Fatty Acids, Medium Chain Fatty Acid

## 1. Introduction

Most foods are considered functional in terms of providing nutrients and energy to sustain daily life, but dietary systems that are capable of preventing or remediating a stressed or diseased state are classified as functional foods [1]. African

Yam Bean or *Sphenostylis stenocarpa* locally known as *Otili* belongs to the Fabaceae family characterized by its fruit (legume) and stipulated leaves. The under-exploited specie is of important food source in Africa, seeds are usually added to soups, made into sauces, or milled into flour [2] [3]. They are grown in countries of West Africa such as Cameroon, Cote d'Ivoire, Ghana, Nigeria, and Togo [4] [5]. It grows as a vine to heights of about 3 m and produces brightly colored flowers in 100 - 150 days [2] [6]. The yam bean is a useful source of nutrients for many African communities with a nutritional value comparable to that of the soybean, although the cooking time for the yam bean is much longer [7] [8]. However, some health problems have been discovered in relation to consumption of this bean including flatulence, stomach cramps, diarrhea and dizziness [5] [9]. Though there are some suggestions of a protective effect of common edible bean, epidemiologic studies are generally insufficient to conclude common edible beans decrease the risk of these diseases [10] [11] [12]. They concluded that they are good source of minor compounds which may have important metabolic and/or physiological effects.

Some of our studies made so far include assessment of the functional properties, nutritional qualities and scavenging ability of the *Otili*-wild beans [13] [14] [15]. Data provided preliminary evidence that consumption of beans diet such as the Nigerian wild—*Otili* (*Sphenostylis stenocarpa*) competed favorably with the common edible bean *Phaseolus vulgaris* in bioactive compounds constituents. The knowledge provided had helped to orient the work of investigators involved in varietal selection and also reduce or eliminate anti-nutritional factors to make non-edible wild bean seeds more acceptable as an inexpensive source of protein. Also in our previous studies, *Otili* expressed higher resistibility ability to the proliferation of colon cancer cells induced by Dextran Sodium Sulphate (DSS) in wister albino rats compared with common edible beans [16].

Despite all these aforementioned findings, this wild underutilized bean is still considered an orphan crop, with a huge untapped potential for improvement both in quantity and quality of food products. In human nutrition, dietary fibers are undegradable by mammalian enzymes, and are therefore potentially available for fermentation by micro-organisms living in the GIT (in humans, mainly the colon). The relationship between some gut flora and humans is not merely commensal (a non-harmful coexistence), but rather a mutualistic relationship [17]. Some human gut microorganisms benefit the host by fermenting dietary fiber into short-chain fatty acids (SCFAs), and medium-chain fatty acids (MCFAs). Microbial metabolic end-products, which account for one third of the metabolites present in the human blood, play an important role in gut homeostasis and have an impact on host metabolism and health [18] [19] [20]. Hence our present study aims at examining the metabolic products formed after fermentation by gut associated microbes in a bid to have insight to the organic products that underlies the chemo-preventive activities of this underutilized wild bean.

## 2. Materials and Method

### 2.1. Collection of Cultivar Media Preparation

The legumes (beans) used in this work is wild-type bean-*Sphenostyles stenocarp* (Otili African yam bean) gotten from the farmers in Ado-Ekiti. Media used are; Nutrient agar, MRS agar, Peptone water. 15 grams of peptone powder was dissolved in 1000 ml of distilled water to prepare peptone water. 5 grams of nutrient agar was dissolved in 178.6 ml of distilled water. 5 grams of the MRS agar was dissolved in 75 ml of distilled water to prepare MRS agar. The media are then sterilized in an autoclave at 121 °C, 15 Pascal for 15 minutes. Nutrient agar slants were also prepared to store used organisms.

### 2.2. Extraction of Non Digestible Fraction

The alkali-catalyzed hydrolysis method as described by Shimin *et al.* [21] was applied to extract the insoluble dietary fiber from the beans sample. The bean samples were prepared by pulverizing using blender. 20 grams of each sample was placed in different beakers in quadruplicate followed by the addition of 25 milliliters of ethyl acetate to each sample. After 3 hours, the slurry was washed with water and dried with hot air at 55 °C overnight. Sodium hydroxide was added at 20 times the volume of the slurry, and the mixture was then centrifuged at 4000 rpm for 15 min. The collected matter was then deposited and washed with water. The insoluble dietary fiber was recovered from the residue after the deposit was washed with 76% ethanol, 95% ethanol and acetone at 4 times the volume of the slurry and dried with hot air at 55 °C overnight. The insoluble dietary fiber content in the final bean samples extract was approximately 40%, while the other 60% of the extract was nitrogen free extract (NFE). The content of IDF was determined according to GB 5009.88-2014.

### 2.3. Isolation of Organisms

*Lactobacillus acidophilus* was isolated from fresh cow-milk using the MRS agar. The other microorganisms *Escherichia coli* and *Bacillus subtilis* used were obtained from the Microbiology Laboratory, Ekiti state University, Ado Ekiti. One loopful from the stocks was dispensed into 9 ml of distilled water and serially diluted in dilution  $10^{-1}$  to  $10^{-7}$ . A loopful was then inoculated into the MRS agar, nutrient agar and peptone water. From the pure culture a loopful were inoculated into the nutrient slant for preservation.

### 2.4. In Vitro Gastrointestinal Fermentation

*In Vitro* colonic fermentation of Total Indigestible Fraction isolated from the bean was fermented in disposable test tubes prepared with Peptone water under strict anaerobic conditions, at 37 °C with slight modification as described by Campos-Vega *et al.*, [12]. A 1:10 (w/v) dilution of selected gastrointestinal microbes (*Escherichia coli*, *Bacillus subtilis* and *Lactobacillus acidophilus*) with 0.1 mol/L, Ph 7 phosphate buffer was prepared and homogenized in a digital

high-speed homogenizer system (IKA-Ultra-Turrax, T18, USA; 1 min, 7847 g). The resulting suspension (1 ml) was distributed in disposable test tubes (containing 9 ml of peptone water), and 0.1 g of the isolated total indigestible fraction from each bean was added. All incubations were performed in triplicate. Each tube was mixed with 100 ml of Sodium hydroxide at room temperature to stop the reaction. The tubes obtained at each time of fermentation were centrifuged (Hermle Z 323 K; Wehingen, Germany) at 35,009 g for 15 min at 4°C. Supernatants were at -80°C until analysis for metabolite profile.

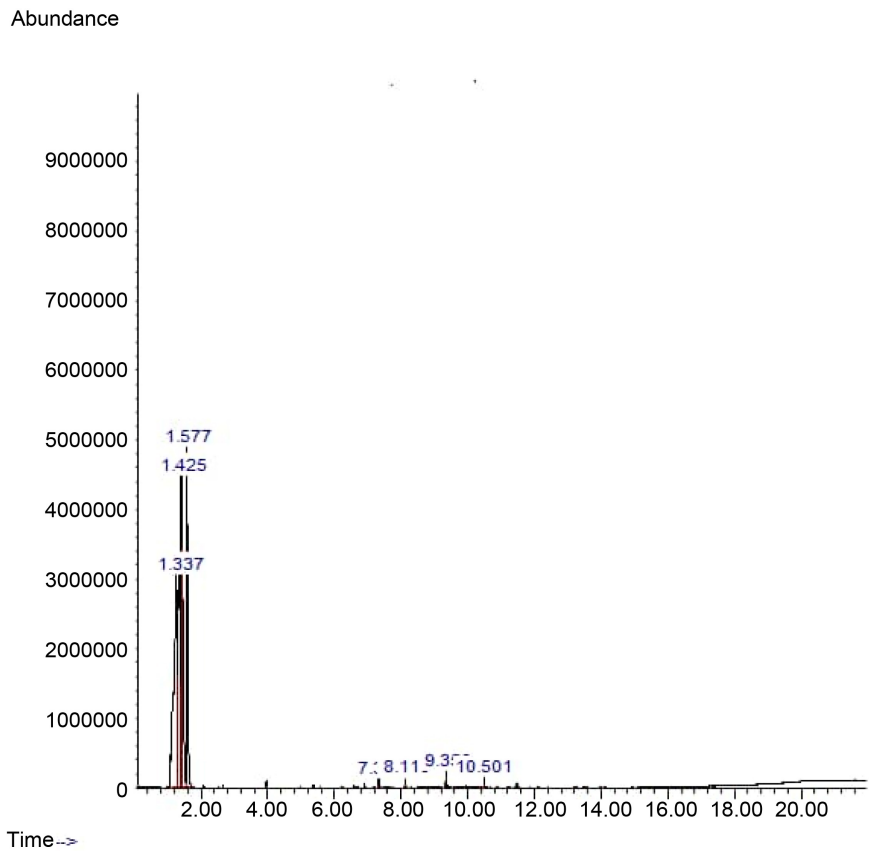
### 2.5. Gas Chromatography-Mass Spectrometry (Gc-Ms) Analysis

The volatile constituents were analyzed with an Agilent 5975C VL mass selective detector coupled to an Agilent 7890A gas chromatograph (Agilent Technologies, Inc., Santa Clara, CA), equipped with a DB-5MS capillary column (60 m 9250 lm 90.25 lm; Agilent). Helium (flow rate, 1 ml/min) was used as the carrier gas. The injector temperature was 250°C. The oven temperature program was 40°C for 5 min, increased by 5°C/min to 200°C and maintained for 2 min, then increased by 20°C/min to 230°C and held for 15 min. Mass spectra were recorded at 70 eV in electron impact (EI) ionization mode. The temperature of the quadrupole mass detector and ion source was 150°C and 230°C, respectively. The injector was used in the splitless mode. SCFA were quantified using standard curves of acetic, propionic, and butyric acids (Sigma-Aldrich). Tentative identification of the volatile components was performed by comparing the mass spectra of the samples with the data system library MSD Chem Station software (Agilent G1701EA version E.02.00.493). Relative concentration of all fermentation metabolites was calculated versus acetic acid as reference expressing the relative proportion of different metabolites, and the results were expressed in µg/ml.

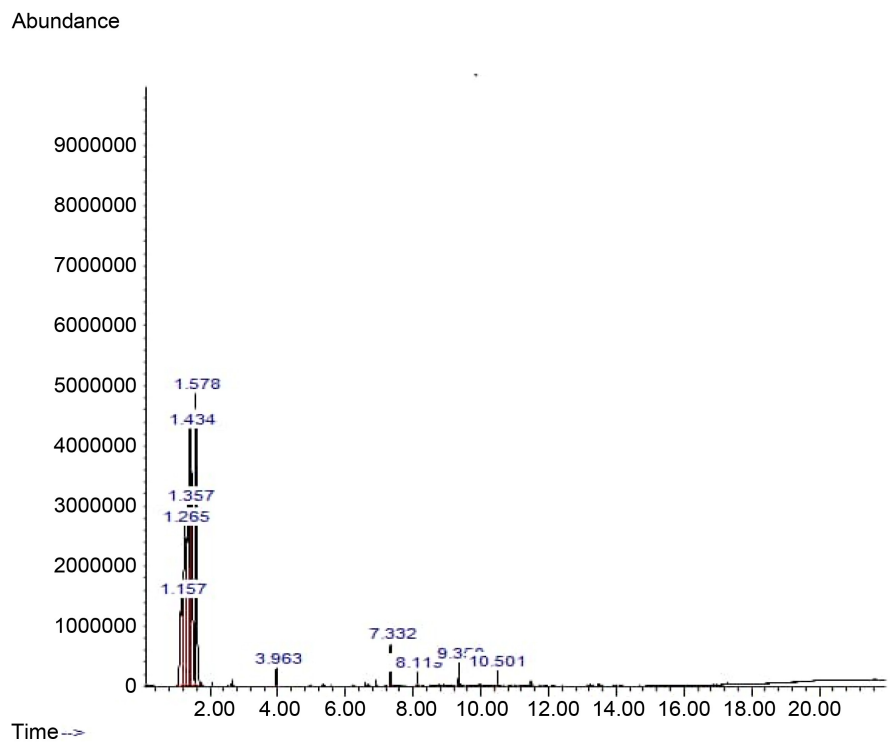
## 3. Results and Discussion

Fermentation is the enzymatic decomposition and utilization of foodstuffs, particularly carbohydrates, by microbes. It takes place throughout the gastrointestinal tract of all animals, but the intensity and products of fermentation depends on number and types microbes, which are generally highest in the large bowel [22]. Chemical changes in foods brought about by enzymes from living microorganisms constitute fermentation. A good balance of intestinal flora is very important to the overall health. Microbial metabolic end-products, which account for one third of the metabolites present in the human blood, play an important role in gut homeostasis and have an impact on host metabolism and health [18] [19] [23].

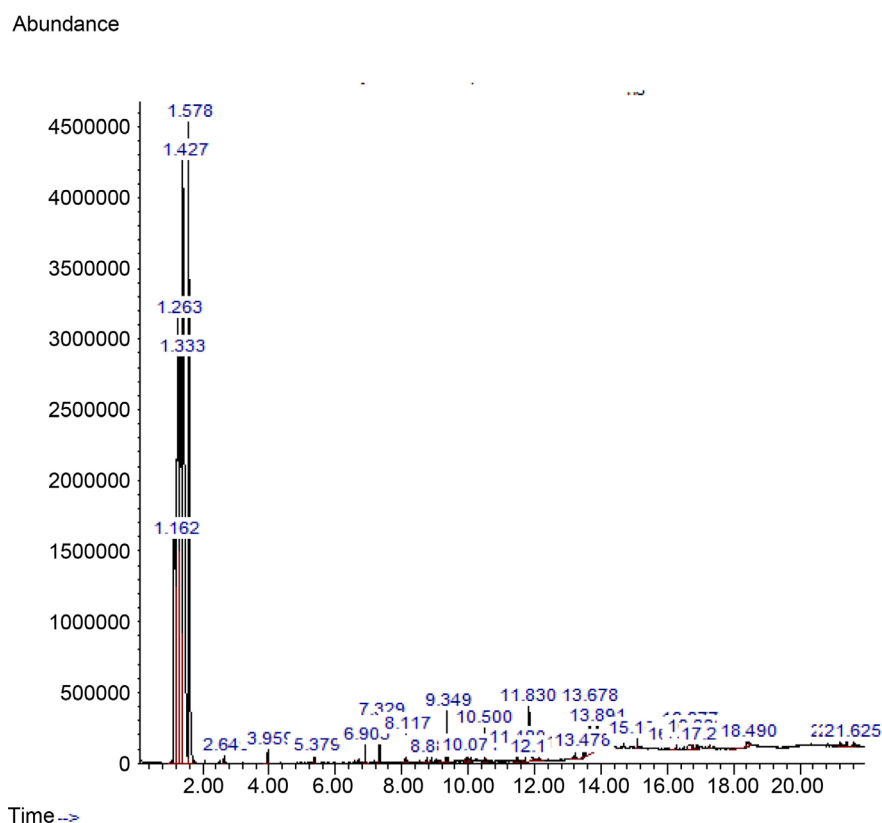
The chromatograms (Figures 1-3) show the mass spectra (µg/ml) of metabolites produced by the three different gastrointestinal microbes respectively after 18 hours of fermentation. A total of fourteen different metabolites belonging to the families of organic acids, short chain fatty acids (SCFAs) and medium chain fatty acids (MCFAs) were detected and evaluated. *Escherichia coli*, *Bacillus*



**Figure 1.** Amounts and types of acids produced in *Otili* by *Escherichia coli*.



**Figure 2.** Amounts of organic acids produced in fermented *Otili* by *Lactobacillus acidophilus*.



**Figure 3.** Amounts and types of acids produced in *Otili* fermented by *Bacillus subtilis*.

*Subtilis*, and *Lactobacillus acidophilus* were able to produce Butyric acid, Hexanoic acid (caproic acid), Dodecanoic acid (lauric acid), 9-Hexadecanoic acid (Palmitelaidic acid), Hexadecenoic acid (palmitic acid). While Octanoic acid (caprylic acid), and Docosenoic acid (erucic) were only detected in both *Escherichia coli*, and *Lactobacillus acidophilus* only at 10.94  $\mu\text{g/ml}$ . The largest amount of fatty acid was produced in form of Hexadenoic acid by the samples fermented by these three fermenting microorganisms within the range 27.26 - 27.39  $\mu\text{g/ml}$ . The second highest metabolite formed was butyric acid which was found highest in *Lactobacillus acidophilus* fermenting medium with value of 19.84  $\mu\text{g/ml}$  while the lowest value of butyric acid was found in *Bacillus subtilis* medium with the value 21.31  $\mu\text{g/ml}$ . Also Lauric acid was found in *Otili* fermented by the three microorganisms with the value 17.85  $\mu\text{g/ml}$ . Caproic acid was found to be very high in *Lactobacillus acidophilus* and *E. coli* with the value 20.87  $\mu\text{g/ml}$  while the lowest value was found in *Bacillus subtilis* with the values 19.02  $\mu\text{g/ml}$ . Caprylic acid was not detected in *Otili* fermented by *Bacillus subtilis* but both *E. coli* and *Lactobacillus acidophilus* fermenting *Otili* had the caprylic acid value 18.06  $\mu\text{g/ml}$ . Capric acid was not detected in both *Lactobacillus acidophilus* and *E. coli* fermenting *Otili* but was found in *Otili* fermented by *Bacillus subtilis* with the value of 21.31  $\mu\text{g/ml}$ . However Tridecanoic acid and Heptadecanoic acid was found in *Otili* fermented by *Bacillus subtilis* only.

It can be rightly established that *Otili* fermented by *Escherichia coli*, *Bacillus*

*subtilis*, and *Lactobacillus acidophilus* contain similar medium chain fatty acids (MCFAs) and short chain fatty acids (SCFAs) as their metabolic products on *Otili* as substrates after 18 hours fermentation. The detection of valuable medium chain fatty acids (MCFAs) such as caproic acid, lauric acid, palmitelaidic acid, palmitic acid caprylic acid, erucic in this study is very interesting aspect of contribution to the existing knowledge. For example rats fed a diet of 19% palmitic acid and 56% carbohydrate for extended periods showed alterations in central nervous system control of insulin secretion and suppression of the body's natural appetite-suppressing signals from leptin and insulin the key hormones involved in weight regulation [24] [25] [26] [27]. It has also been established also that caprylic acid acts as a non-competitive  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist at therapeutically relevant concentrations, in a voltage- and subunit-dependent manner, and this is sufficient to explain its antiseizure effects [28]. This directs inhibition of excitatory neurotransmission by decanoic acid in the brain contributes to the anti-convulsant effect of the MCT ketogenic diet. Decanoic acid and the AMPA receptor antagonist drug act at separate sites on the AMPA receptor, and so it is possible that they have a cooperative effect at the AMPA receptor, suggesting that the ketogenic diet could be synergistic [29] [30] [31]. In-vitro experiments have suggested that some fatty acids including lauric acid could be a useful component in a treatment for acne, but no clinical trials have yet been conducted to evaluate this potential benefit in humans [30] [31]. Lauric acid increases total serum cholesterol more than many other fatty acids. But most of the increase is attributable to an increase in high-density lipoprotein (HDL) (the "good" blood cholesterol). As a result, lauric acid has been characterized as having "a more favorable effect on total HDL cholesterol than any other fatty acid examined, either saturated or unsaturated [32] [33] [34]. Early Studies on laboratory animals showed that erucic acid appears to have toxic effects on the heart at high enough doses [35] [49]. An association between the consumption of rapeseed oil and increased myocardial lipidoses, or heart disease, has not been established for humans [37] [38]. While there are reports of toxicity from long-term use of Lorenzo's oil which contains erucic acid and other ingredients [39], there are no reports of harm to people from dietary consumption of erucic acid [36] [40] [41]. Caprylic acid is used commercially in the production of esters used in perfumery and also in the manufacture of dyes. Caprylic acid is an antimicrobial pesticide used as a food contact surface sanitizer in commercial food handling establishments on dairy equipment, food processing equipment, breweries, wineries, and beverage processing plants. It is also used as disinfectant in health care facilities, schools/colleges, animal care/veterinary facilities, industrial facilities, office buildings, recreational facilities, retail and wholesale establishments, livestock premises, restaurants, and hotels/motels. In addition, caprylic acid is used as an algaecide, bactericide, fungicide, and herbicide in nurseries, greenhouses, garden centers. Products containing caprylic acid are formulated as



soluble concentrate/liquids and ready-to-use liquids [42] [43]. For ghrelin to have a hunger-stimulating action on a hypothalamus, caprylic acid must be linked to a serine residue at the 3-position of ghrelin. To cause hunger, it must acylate an –OH group. Other fatty acids in the same position have similar effects on hunger. No wonder, consuming beans may contribute to feelings of short-term satiety as a result of the beans' fiber and protein content [32].

However, *Lactobacillus acidophilus* had the highest production of butyric acid; the notable short chain fatty acids detected in this present study, *Bacillus subtilis* had the least production of butyric acid while there was no trace of other volatile SCFAs such as lactic, acetic, and propionic acid. The long 18 hour fermentation period may be responsible for this, the volatile short chain fatty acids metabolites may have been used up or converted to the detected MCFAs. This corroborates the submission of Marques *et al.*, [44] stating that involvement of *Lactobacillus acidophilus* in fermentation process will increase the production of butyric acid. Russell *et al.*, [45] stated that lactic acid produced in-vitro by lactic acid bacteria, is used by some strictly anaerobic butyrate-producing bacteria for the production of high concentrations of butyric acid. In further study they submitted that an increase in butyrate production may result from a direct stimulation of butyrate producers or indirect effects such as metabolic cross-feeding of fermentation products from other bacterial groups [46] [47]. Butyric acid, produced within the intestinal lumen by bacterial fermentation of dietary carbohydrates, exerts a wide variety of effects on intestinal function [48]. First of all, butyric acid is the preferred source of energy for colonocytes. It affects cellular proliferation, differentiation and apoptosis. Moreover, butyric acid has well documented anti-inflammatory effects. Inhibition of histone deacetylase activity, resulting in hyperacetylation of histones, and as a consequence suppression of nuclear factor kappa B activation, is a likely explanation. Secondly, it has been proposed that butyric acid reinforces the colonic defense barrier by increasing production of mucins and antimicrobial peptides. Thirdly, it has been shown that butyric acid decreases intestinal epithelial permeability by increasing the expression of tight junction proteins [49] [50] [51]. These are thus probiotic candidate for the treatment of Inflammatory Bowel Diseases.

#### 4. Conclusion

From this study, it can be deduced that certain microorganisms contribute to the breakdown of indigestible fraction of beans thereby improving the production of SCFA and MCFA. These data provided preliminary evidence that consumption of beans diet such as the wild-*Otili* improves proper functioning of the colon.

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### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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