In Vivo Studies on the Prebiotic Effects of *Vernonia amygdalina* Leaf Extracts

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Abstract

The aqueous leaf extract of Vernonia amygdalina was evaluated for its prebiotic effects on seven human volunteers, made up of five men and two women. The evaluation was conducted by monitoring the effects of oral administration of the extract on total and differential counts of aerobic faecal bacteria, bowel changes and other physiological parameters. Three main bacterial types (Enterococcus sp. Lactobacillus sp. and Corynebacterium sp.) were isolated from the stool samples of the subjects. Enterococcus sp. was selectively stimulated over the other species by the extract and increase in numbers of Lactobacillus appeared to be dependent on the metabolic activities of Enterococcus. The subjects reported changes in bowel function including increased faecal volume, softening of stool and flatulence; effects which have been associated with known prebiotics. Evaluation of possible antagonism between the intestinal bacteria and other bacteria showed that while there was no antagonism between the three faecal isolates, Lactobacillus exhibited antagonism against intestinal pathogens such as Escherichia coli, Salmonella, Staphylococcus, Shigella and Klebsiella. The results from this study show convincingly that aqueous leaf extracts of V. amygdalina contains prebiotics. Furthermore there is a strong suggestion that oral administration of this extract may enhance the production of vitamins, particularly folate, in the intestinal environment by intestinal bacteria.

Keywords: Prebiotic, *Vernonia amygdalina,* Enterococcus, Lactobacillus, Folate

Introduction

Prebiotics are non-digestable food ingredients that selectively stimulate the growth of a limited number of beneficial microorganisms in the intestinal tract to improve host health (Gibson and Roberfroid, 1995; Cummings *et al.,* 2001; De Vuyst *et al*., 2005; Rastall *et al*., 2005).

For decades, the roles of the intestinal microflora in maintaining the structure and function of the intestine have been documented. These roles include those of digestion, fermentation, inhibition of pathogenic organisms, provision of enzymes and amino acids, and development of immunity (Macfarlane and Cummings, 1999; Matsuki *et al*., 1999; Cummings *et al*., 2001; Morelli *et al*., 2003; Tannock *et al*., 2004; Rastall *et al*., 2005). Interest in these beneficial roles of the intestinal microflora has led to the development of various types of probiotic foods, that is, foods containing live microorganisms; for example, yoghurt (Macfarlane and Cummings, 1999). The use of probiotics has, however, been faced with a serious problem of consumer confidence because many individuals are skeptical about consuming live microorganisms, regardless of the propounded benefits. Also, some such organisms have been known to cause disease in immunocompromised individuals (Macfarlane and Cummings, 1999). Consequently, attention has recently shifted from probiotic to prebiotic studies. The most commonly studied and available prebiotics are the oligosaccharides, galactooligosaccharides and pectic oligo-saccharides (Menne *et* al., 2000; Cummings *et al*., 2001; Morelli *et al*., 2003; Rastall *et al*., 2005; De Vuyst *et al*., 2005; Saulnier *et al*., 2007). These complex carbohydrates are non-digestable by the gastric juice, pancreatic and brush border enzymes and selectively stimulate the growth of intestinal microflora. These carbohydrates are commonly found in fruits, vegetables and plant products (Menne *et al.,*2000; Rastall *et al*., 2005).

This study aimed at evaluating the prebiotic effects of *Vernonia amygdalina* leaf extracts. The leaves are commonly used in Nigeria especially in Igbo traditional medicne for treating various illnesses including gastrointestinal disturbances. *Vernonia amygdalina*, commonly called “bitterleaf” in English and “Olubu or Onugbo” in Ibo is a small shrub that grows predominantly in tropical Africa. There have been several reports on its antimicrobial, antiplasmodial, antitumor, antioxidant and antihelminthic properties (Jisaka *et al*., 1993; Izevbigie, 2003; Farombi, 2003; Ehiagbonare, 2007), but no reports yet on the prebiotic effects, which was investigated in this study.

The specific objectives of the study were: to evaluate the effects of the *V. amygdalina* aqueous extract on total and differential counts of the intestinal microflora; to isolate and characterize the organism (s) that is (are) stimulated by the extract; and to study, *in vitro,* possible antagonistic interactions between the organisms of the microflora and between the microflora and some other organisms including intestinal pathogens.

Materials and Methods

Test plant: The leaves of the plant, *Vernonia amygdalina,* used for this study were obtained from a private farm in Nsukka and authenticated by a botanist from the Department of Botany, University of Nigeria, Nsukka.

Human volunteers: Twelve volunteers, made up of seven males and five females were randomly recruited from the student population of University of Nigeria, Nsukka. Subjects were asked to provide information on their general health. Only individuals having no history of gastrointestinal disease and who were not using any antibiotics for at least three months prior to the commencement of the study, were selected. Informed consent was obtained from the volunteers according to the ethical guidelines of the University of Nigeria, Nsukka.

Extraction procedure: The leaves were washed in clean water and dried. The dried leaves were crushed into a homogenous powder using mortar and pestle. One hundred grams (100 g) of the powder was soaked in 1 L of water for 24 h with intermittent stirring. The aqueous extract was then filtered through a 2 mm mesh filter and the filtrate stored at 4^{o}C in clean sterile bottles.

Effect of the extract on human intestinal flora: This phase of the study was carried out over a six-week period. In the first stage, lasting two weeks, no extract was administered, while the faecal flora of the subjects was monitored. This period was called the pretreatment period. In the second stage of another two weeks, 150 ml of the extract, given in form of a drink, was administered once daily, usually with or immediately after supper. The changes in type and numbers of the faecal flora were monitored. This period was called the treatment period. During the last stage, lasting another two weeks, the extract was again excluded while monitoring of the faecal flora continued. This period was called the post-treatment period. The subjects were given strict instructions to eliminate fruit juices, soft drinks, yoghurt, alcohol and antibiotics from their diets for the duration of the study, but water and other daily diets were allowed.

Collection and processing of faecal samples: Fresh stool samples were obtained every three days from the subjects throughout the study period. All samples were processed aerobically and microaerophilically (in anaerobic jars) within 3 h of collection. Each faecal sample was homogenized (10%, w/v) in sterile phosphate buffered saline comprising (w/v): sodium chloride, 0.8%; potassium phosphate, 0.2%; sodium phosphate, 0.115%; at pH 7.4. Following homogenization, dilutions of faecal suspension were inoculated onto agar media including Nutrient, De Man, Rogosa and Sharpe (MRS) agars (Oxoid). Cultures were prepared in triplicates and incubated both aerobically and microaerophilically at 37^{o}C for 48 h.

Enumeration of intestinal organisms: Colonies resulting from the 48 h faecal cultures were enumerated and characterized based on cultural, morphological and biochemical characteristics as outlined in *District Laboratory Practice in Tropical Countries* (Cheesbrough, 2004) and Manual of Clinical Microbiology (Balows *et al.,* 1991). Organisms were identified to at least genus level.

Evaluation of bowel functions: The changes in bowel function as well as general health conditions of the subjects were monitored throughout the study by oral interview of the subjects as well as visual examination of the faecal samples.

Antagonistic evaluation of isolates: The faecal isolates were evaluated for antagonistic properties against each other and against other bacteria, including *Salmonella* sp., *Staphylococcus aureus*, and *Escherichia coli*. Each isolate was inoculated unto Nutrient or MRS agar and incubated for 24 h at 37^{o}C. Then, a soft agar, at 42 – 45^{o}C, mixed with the test organism, was overlaid on the 24 h culture. The co-culture was then incubated for another 24 h. Thereafter, inhibition zones of the test organisms were observed and measured.

Statistical analysis: The counts of faecal organisms were analyzed for differences due to the consumption of the test extract. Analysis of Variance (ANOVA) and Least Significant Difference (LSD) determinations were used to test for differences between baseline bacterial counts during the pretreatment period, increases during the treatment period and post-treatment periods. Differences were considered significant at $P < 0.05$.

Results

Human volunteers: Twelve volunteers, including seven males and five females were initially recruited for the study. However, five people (two males and three females) opted out before the completion of the study. Consequently, results were obtained from only seven subjects.

Enumeration of intestinal organisms: A total of eight organisms were isolated from the stool samples of the subjects. These faecal bacteria were identified as *Staphylococcus aureus, Bacillus cereus, Lactobacillus* sp., *Enterococcus* sp., *Corynebacterium* sp., *Proteus* sp., coagulase negative *Staphylococcus* sp. and *Pseudomonas aeruginosa.* Some of the species occurred in an irregular pattern and in low numbers during different phases of the study and were regarded as transient flora, while others occurred consistently throughout all phases of the study. These were regarded as the resident intestinal flora. These members of the resident flora were *Lactobacillus* sp., *Enterococcus* sp. and *Corynebacterium* sp. The relative abundance of the different organisms through the different phases of the study are shown in Table 1.

Effect of consumption of extract on bacterial counts: The numbers of organisms per gram of stool of the three members of the intestinal microflora were monitored for all seven subjects. Similar patterns were observed for all seven subjects and the results for four of the subjects are shown in Figures 1 to 4. There was a noticeable increase in the number of enterococci, beginning within 72 h following administration of the extract. The numbers rose steadily throughout the treatment period, declining with ceasation of treatment (Figures 1 – 4).

Table 1. Relative abundance of organisms isolated from stool samples during the different periods of the study

Period Isolated/ Organisms	Pretreatment				Treatment				Post-treatment			
	Day 0	Day 3	Day 7	Day 11	Day 14	Day 18	Day 22	Day 26	Day 29	Day 33	Day 37	Day 41
Staphylococcus aureus	+	+	+	+	-	-	-	-	-	-	-	-
Bacillus cereus	+	+	+	+	-	-	+	-	-	-	-	+
***Lactobacillus* sp.**	+	+	+	+	+	+	++	++	+++	+++	+++	+
***Enterococcus* sp.**	+	++	++	++	+++	+++	+++	+++	+++	+++	+++	++
***Corynebacterium* sp.**	+	+	++	++	+++	+++	++	++	+	+	+	+
***Proteus* sp.**	+	+	+	-	-	-	-	-	-	-	+	+
Coagulase negative *Staphylococcus*	-	-	-	-	-	-	-	-	-	-	+	+
Pseudomonas aeruginosa	-	-	-	+	+	+	-	-	-	-	-	-

Key: - *= not isolated, + = numbers < 10^6 cfu/g of stool, ++ = 10^6 – 10^7 cfu/g of stool, +++ = 10^7 - 10^{10} cfu/g of stool*

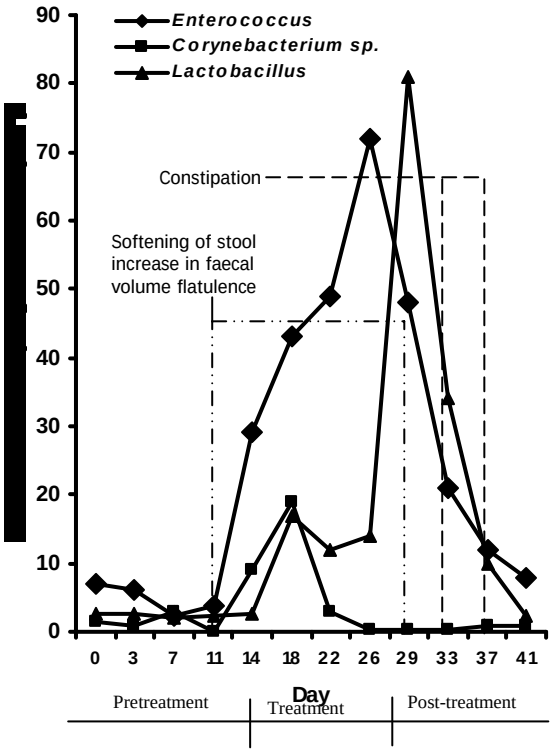

Fig. 1: Effect of oral administration of *V. amygdalina* extract on bacterial numbers

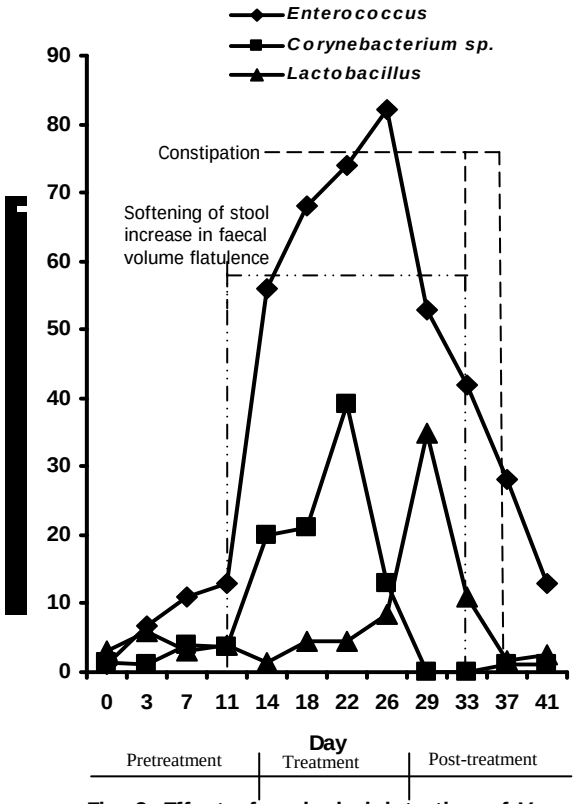

Fig. 2. Effect of oral administration of *V. amygdalina* aqueous extract on bacterial counts in Subject 2

Statistical analysis proved this increase to be significant at $P < 0.05$. The increase in number of *Corynebacterium* was more gradual with noticeable increases recorded from about the third day, reaching a peak by around the seventh day of treatment, before declining and dropping to base levels with ceasation of treatment (Figures 1 – 4). These increases were not significant ($P > 0.05$). There was a delay in the increase in numbers of lactobacilli, with the rise starting only after the peak of enterococci. Thus, the increase in number of lactobacilli was recorded immediately following ceasation of treatment (Figs. 1 – 4). The increase in numbers was significant ($P < 0.05$) only in the post-treatment period.

Change in bowel and physiological functions: Oral interview of the subjects revealed that most observed a softening of the stool during a few days following treatment. There was increase in faecal volume, increase in appetite and some individuals reported experiencing a sedative effect during treatment. In addition to these, most of the subjects reported experiencing a brief period of constipation. It was noteworthy that these periods coincided with the days following the peak of *Lactobacillus* in each of the subjects (Figs. 1 – 4). Also, some individuals experienced mild flatulence during treatment (Figs. 1, 3 and 4).

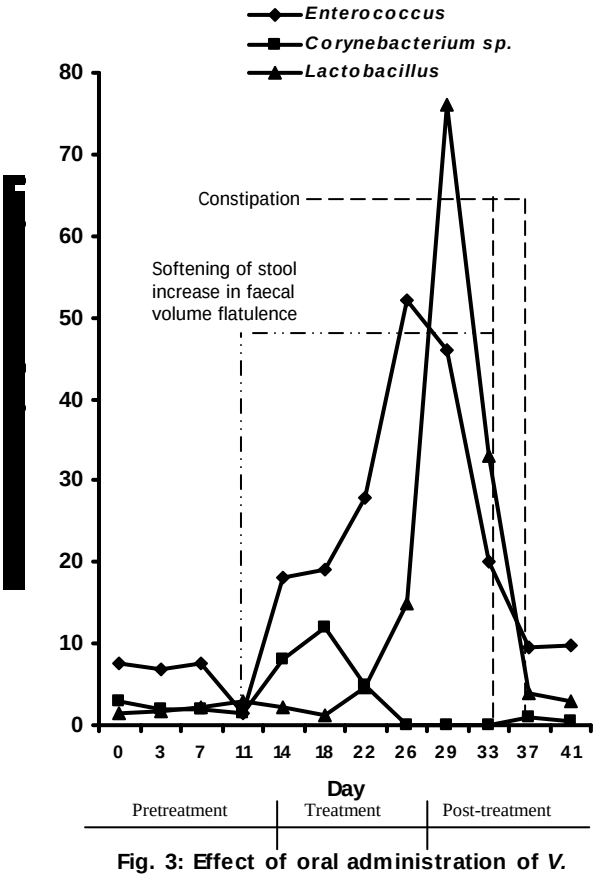

Fig. 3: Effect of oral administration of *V. amygdalina* aqueous extract on bacterial counts in subject 3

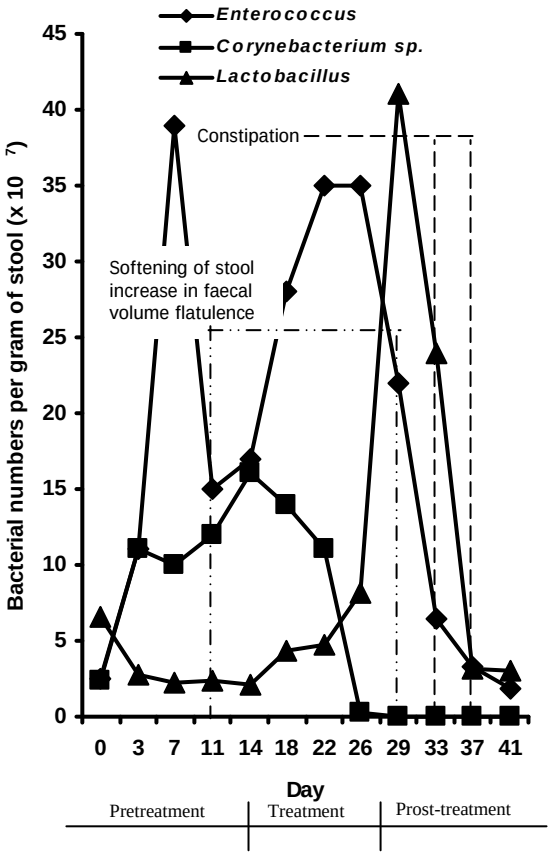

Fig. 4: Effect of oral administration of *V. amygdalina* aqueous extract on bacterial counts in Subject 4

Interactions between faecal isolates and test organisms: Evaluation of possible antagonistic interactions between the intestinal flora and selected intestinal pathogens showed that only *Lactobacillus* exhibited antagonism against the pathogens (Table 2).

Discussion

Three bacterial types were found to be consistently present in the stool samples of the human subjects during the eleven-day monitoring period (pre-treatment) and were therefore considered to be members of the resident intestinal microflora. The morphological and biochemical characteristics of these organisms were consistent with those of *Enterococcus, Corynebacterium* and *Lactobacillus.* The three organisms were monitored for changes in numbers through the pre-treatment, treatment and post-treatment periods. The results showed that there were changes in the numbers of cells of the organisms per gram of stool through the different periods of study. Analysis of the data by one-way ANOVA and LSD, showed that the numbers of *Enterococcus* per gram of stool, were significantly ($P < 0.05$) higher during the treatment period and a few days following ceasation of treatment. The numbers of *Lactobacillus* increased significantly ($P < 0.05$) in the first few days after the ceasation of treatment, while there was no significant ($P > 0.05$) difference in the numbers of *Corynebacterium* in the different periods. These results suggest that, of the three organisms, only *Enterococcus* was directly stimulated by the treatment.

Enterococcus has previously been listed as one of the probiotic bacteria active in the gut (Macfarlane and Cummings, 1999; Crittenden *et al*., 2003). It has been established that probiotic bacteria active in the intestinal tract may be stimulated by consumption of certain foods or food ingredients (prebiotics). The stimulation of *Enterococcus* in this study, over other identified species, suggests that the aqueous extract of *V. amygdalina* leaves contains prebiotic constituents. Also, the pattern of increase in bacterial numbers, with *Lactobacillus* coming consistently last, may suggest that prebiotic substances present in the extract were metabolized by the other organisms to release or produce a compound or compounds, which were required for the growth of *Lactobacillus*.

Table 2: Interactions between the faecal isolates and selected test bacteria

Faecal Isolates	Test Organisms						
	E. coli	*Salmonella* sp.	*Staphylococcus* sp.	*Shigella* sp.	*Klebsiella* sp.	*Enterococcus* sp.	*Corynebacterium* sp.
***Enterococcus* sp.**	-	-	-	-	-	NT	-
***Corynebacterium* sp.**	-	-	-	-	-	-	NT
***Lactobacillus* sp.**	+	+	+	+	+	-	+

Key: - *= No inhibition, + = Inhibition, NT = Not tested*

This is in line with the reports of Crittenden *et al.* (2003), which suggested that probiotic bacteria, when stimulated by consumption of certain foods may produce vitamins, particularly folate, which is utilized by lactobacilli.

Folate is widely distributed in the biological world, intestinal bacteria being one source of the vitamin. Faecal bacteria, which have been reported to produce folate include *Enterococcus faecium, Streptococcus thermophilus* and various species of *Bifidobacterium* (Crittenden *et al*., 2003; Holasova *et al*., 2004; Pompei *et al*., 2007). There have previously been questions regarding the extent to which these probiotic bacteria can contribute to the folate requirement of colonic epithelial cells. However, experiments by Pompei *et al*. (2007) demonstrated that addition of *Bifidobacterium adolescentis* to the diets of some human volunteers increased the folate concentration in the colonic environment. Although there were no attempts to assay for production of vitamins in this study, there were complaints of constipation by the volunteers for a few days in the post-treatment period, coincidental with the period immediately following the peak of *Lactobacillus.* Constipation has been associated with shortage or deficiency of folate or folic acid (Clark, 2008; Whiting, 2008). It is possible that rapid consumption of folate by the increased numbers of lactobacilli resulted in the temporary occurrence of constipation. Most species of *Lactobacillus* have been shown to deplete folate concentrations in growth media (Crittenden *et al*., 2003). Lactic acid bacteria such as *Lactobacillus* have a strict growth requirement for folic acid (Hugenholtz *et al*., 2002).

In the large intestine, prebiotics, in addition to their selective stimulation of the intestinal microflora, influence many aspects of bowel function through fermentation. They affect bowel habit, are mildly laxative and have a propensity to produce flatulence (Cummings *et al*., 2001). The results of the oral interview of the volunteers in this study, showed that the volunteers experienced various changes in bowel function during the treatment period consistent with effects reported for prebiotics. The softening of stool and increase in faecal volume reported by the volunteers in this study are all part of the laxative effect reported for prebiotics. According to Cummings *et al.* (2001), the prebiotics produce their laxative effect via stimulation of microbial growth, increase in bacterial mass, and thus, stimulation of peristalsis by the increased bowel content. The flatulence is due to the production of carbon dioxide and hydrogen, which are two of the major products of prebiotic metabolism, others being short chain fatty acids (SCFAs) and bacterial cell mass (Cummings *et al*., 2001).

Another established role of the human microflora in health is that of inhibition of various human pathogens. By extension, therefore, stimulation of the intestinal microflora by whatever mechanism, should provide the human involved with some protection against intestinal pathogens. To evaluate this potential benefit, the intestinal species stimulated by the extract in this study, were tested for antagonism against each other and some selected intestinal pathogens. The results showed that only *Lactobacillus* exhibited antagonism against the pathogens. This is in line with the results from a study by Clements *et al*. (1981) in which different organisms including lactobacilli, bifidobacteria, enterococci and staphylococci were tested for prophylactic effectiveness against traveller's diarrhoea and *Lactobacillus* but not *Enterococcus* was one of the organisms that reduced diarrhoea.

The results from this study show that *V. amygdalina* leaf extract contains prebiotics, which stimulate selected intestinal bacteria, influencing bowel functions. Furthermore, there is a strong suggestion that oral administration of this extract may enhance the production of vitamins, particularly folate, in the intestinal environment by intestinal bacteria. This study therefore highlights another important use of the *Vernonia amygdalina* plant for human health. The *V. amygdalina* leaves, which are commonly used in many parts of Eastern Nigeria for soup (bitterleaf soup) can now be recommended for their health benefit, particularly for pregnant and nursing women.

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