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Microbial interaction in selected fermented vegetable condiments in Nigeria

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Abstract

Fermented condiments remain the key constituents of diets throughout the world especially in Africa and Asia. The process of fermentation of these condiments involves different types of microorganisms which interact with each other and mode of interaction need to be understood. Iru and ogiri-egusi were purchased from retail markets in Oyo State, Southwest Nigeria. Microorganisms were isolated, characterised and co – cultured for fermentation of the condiments. Bacteria obtained were 168; 100 Gram-positive bacteria, 30 Gram-negative and 38 Lactic acid bacteria. *Bacillus* species comprising *B. subtilis, B. pumilus, B. licheniformis* and *B. megaterium* had the highest frequency of occurrence of 72% while *Staphylococcus epidermidis* and some lactic acid bacteria were consistently present. *B. subtilis* had the highest growth rate at 60 h when used singly both in iru and ogiri-egusi. Co – culturing *B. subtilis* and *S. epidermidis* in iru and ogiri-egusi showed an increase in growth rate of 46 and 23% respectively while addition of *L. plantarum* gave a decrease of 33% in growth thus depressing the growth of *B. subtilis*. The factors at play by the lactic acid bacteria during the interaction were presumed to be due to production of acids and metabolites which have effect on the proteolytic *Bacillus* species. Knowledge of the key roles of the organisms studied facilitates the development of starter cultures using mixed cultures.

Introduction

Fermentation can be regarded as one of the oldest technologies for the production of food products with desirable properties such as extended shelf life and good organoleptic properties (Smid and Hugenholtz, 2010). Condiment is a substance used to enhance the flavour of food. It is the official nomenclature adopted by the International Organisation for Standardisation. Fermented condiments remain the key constituents of diets throughout the world especially in Africa. These condiments are usually obtained from seeds of legumes which account for up to 80% of dietary protein for some groups of people within the society. Condiments are prepared by traditional methods of uncontrolled solid substrate fermentation resulting in extensive hydrolysis of the protein and carbohydrates components (Fetuga *et al*., 1973; Achi, 2005). The fermentation is usually carried out in a moist solid state, involving contact with appropriate inocula of assorted microorganisms and is accomplished by the natural temperature of the tropics. The desired state of fermentation of the condiments is indicated by the formation of mucilage and overtones of ammonia produced as a result of the breakdown of amino acids during the fermentation (Odunfa, 1986). Some of the most important food condiments are "iru" from African Locust bean (Odunfa, 1986), "ogiri" from fermented melon seed (Barber and Achinewhu, 1992), "daddawa" from soybean Omafuvbe *et al*., 2000, 2002) "ugba" from oil bean seeds (Obeta, 1985), "ogiri – igbo" from castor oil seeds (Odunfa, 1986), "owoh" from cotton seeds (Sanni and Ogbonna, 1991).

The process of fermentation in any food matrix is a complex microbiological process involving interactions between different types of microorganisms (Sieuwerts *et al*., 2008). The contribution of the various microbial floras during fermentation is determined by the substrate composition and hygiene during production. Microorganisms in natural ecosystem coexist with and live in symbiotic relationship with other organisms. These natural symbiotic relationships are usually very stable for long period of time (Masato *et al*., 2008). The ability of a particular microorganism to dominate a fermentation process depends on several factors and sometimes microorganisms initially present in very low number during the fermentation process will outnumber the other organisms thereby inhibiting their growth. Microorganisms use the nutritional components of the seeds during fermentation thereby

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converting them into products that contribute to the chemical composition and taste of the condiments. An appreciable number of *Bacillus* sp. have been isolated from various fermented food condiments, yeasts and other species of bacteria have also been isolated which include *Staphylococcus* sp., proteolytic and salt tolerant microorganisms. Use of consortia in fermented foods are frequently complex, involving multiple layers of similar looking microorganisms that often have complimentary physiological properties (Sieuwerts *et al*., 2008). The process of natural and industrial food fermentation are driven by either simple or complex communities of microorganisms. The microorganisms will not only interact with the fermentable substrate but also with each other (Smid and Lacroix, 2013). The complex microbial consortia perform more complex activities and tolerate more variations in the environment as compared to pure cultures. This is so because, members of the consortium communicate with one another by trading metabolites or by exchanging molecular signals thereby each individual cell in the mixture responds to the presence of others in the consortium (Keller and Surette, 2006) there is a division of labour between the microbes in the consortium leading to an overall output that can only be explained by combining tasks performed by constituent individuals (Ayad *et al*., 2001). Microbial interaction in fermented foods involve the combined activities of microbial successions that are associated with changes in the environmental conditions created by the metabolic activities of the microorganisms involved in the fermentation process (Smid and Hugenholtz, 2010). The type and number of microorganisms originally present in the substrate as well as the composition of the raw materials influence these overlapping activities prevailing environmental conditions in the substrate shift the activities of the microflora to either the useful type or the one that can cause spoilage of the product (Sieuwerts *et al*., 2008). It is the predominant microorganisms that change raw materials into entirely new ones. The type of interactions could be competition, mutualism, commensalism, amensalism and parasitism (Smid and Lacroix, 2013). The aim of this work is to identify the type of interactions among different species of microorganisms involved in the fermentation system of condiments so as to estimate the growth rate of single and mixed culture in fermented condiments.

Materials and Methods

Collection of samples

Samples used are iru and ogiri egusi. They were purchased in retail markets in Ibadan, Oyo State, Nigeria at six different occasions. They were collected in sterile polythene bags and were transported to the laboratory for further work.

Isolation of microorganisms

Tenfold serial dilution was used for the isolation. Ten gram of fermented food was weighed into 90 ml sterile distilled water. One millilitre of 105 and 106 dilutions of the various samples were plated out using the pour plate method of Harrigan and McCance, 1966. Isolation of microorganisms was done using media such as Nutrient agar, Malt Extract agar, MacConkey agar and Potato Dextrose agar (PDA) and MRS agar for isolation of *Lactobacillus* sp. Inoculated plates of Nutrient agar and MacConkey agar was incubated at 30◦C for 24 h. Malt Extract and PDA plates were incubated at room temperature for 72 h. All the plates were incubated aerobically.

Characterisation of Isolates

Taxonomic studies were carried out on purified isolates based on their cultural, morphological, biochemical and physiological characteristics.

Preparation of substrate

Iru (locust bean): African locust beans were purchased from Bodija market, Ibadan. The seeds were cooked for 90 min using pressure pot. The seed coats were removed by pressing the seeds between hands and the cotyledon was separated from the seed coats after washing and rinsed with several changes of clean tap water. The cotyledons obtained were distributed into sterile Erlenmeyer flasks.

Ogiri egusi (Melon seed): Shelled melon seeds obtained from the market were sorted out to remove grit, dirt and decomposing seeds. The seeds were washed with tap water and then boiled with pressure pot for 2 h, Water used was decanted and melon seeds were then distributed into sterile Erlenmeyer flasks.

Preparation of Inocula

Representative colonies were inoculated into sterile broth and were incubated at 30◦C for 24 h. Each of the cultured broth was serially diluted out and one millilitre of 10^4 dilution was plated out using pour plate method to determine the approximate number of cells present in the dilution.

Inoculation of Substrates

In 50 mL Erlenmeyer flask, 5 g of substrate was placed and 10 mls of sterile distilled water and mixed thoroughly. Then 1ml of the representative isolates was used to inoculate the substrate. The substrate

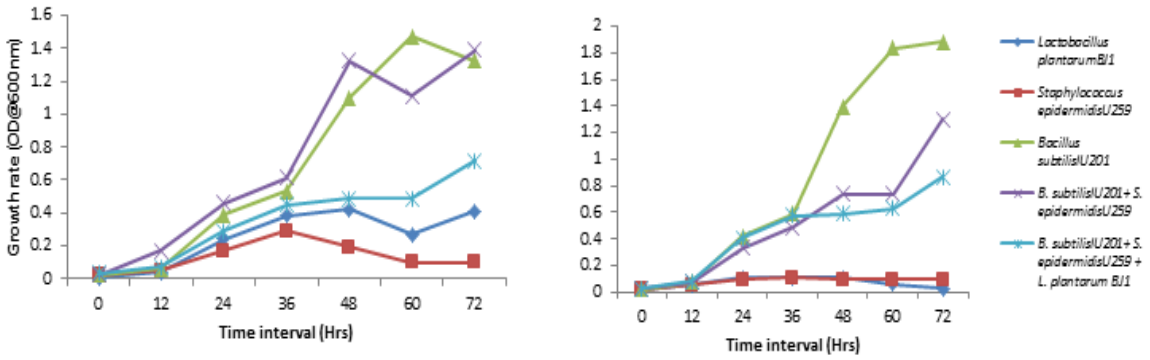

Figure 1. Growth rate of *Bacillus subtilis, Staphylococcus epidermidis* and *Lactobacillus plantarum* in "iru" and "ogiri egusi"

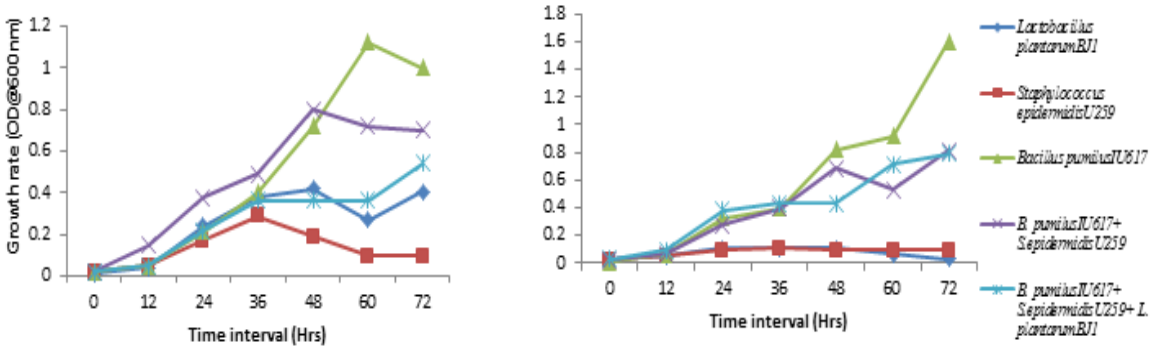

Figure 2. Growth rates of *Bacillus pumilus, Staphylococcus epidermidis* and *Lactobacillus plantarum* in "iru" and "ogiri egusi"

culture mixture was incubated aerobically at 35◦C for different time intervals (0, 12, 24, 36, 48, 60 and 72hrs). Growth was measured using Spectrum lab 752S UV VIS spectrophotometer at 600 nm using distilled water as blank.

Growth of mixed culture in substrate

One millilitre of 10^4 dilutions of *Bacillus* sp. and *Staphylococcus epidermidis* with 1 ml of 102 of *Lactobacillus* sp. was used as mixed culture to inoculate 5 g of different substrates in 10mls of sterile distilled water. The substrates were incubated aerobically at 30◦C for 0 – 72 h and growth measured using a Spectrum lab 752S UV VIS spectrophotometer at 600 nm.

Results

A total of 168 organisms were obtained from this study which comprises of 100 Gram positive bacteria, 30 Gram negative bacteria and 38 Lactic acid bacteria. After characterisation based on their microscopic, biochemical and physiological characteristics, they were classified as *Bacillus subtilis* (44), *B. licheniformis* (20), *B. megaterium* (8), *B. pumilus* (4), *B. polymxa* (6), *B. cereus* (9), *B. coagulans* (3), *Staphylococcus epidermidis* (3), *S. aureus* (1), *Micrococcus luteus* (2), *Salmonella* sp. (1), *Escherichia coli* (12), *Citrobacter freudi* (3), *Proteus mirabilis* (1), *P. vulgaris* (2), *Enterobacter aerogenes* (7), *Klebsiella aerogenes* (3), *Paracoccus denitrificans* (1), *Lactobacillus brevis* (10), *L. fermentum* (3), *L. casei* (5), and *L. plantarum* (20).

The growth rate using single and mixed culture of *B. subtilis* IU201, *S. epidermidis* U259 and *L. plantarum* BJ1 in iru and Ogiri egusi is shown in Figure 1. It was observed that *B. subtilis* IU201 had its optimum growth concentration at 60 h when inoculated on iru alone, when it was co-cultured with *S. epidermidis* U259, the highest growth was observed at 48 h, it declined at 60 h and later increased after 72 h. Consortium of *B. subtilis* IU201, *S. epidermidis* U259 and *L. plantarum* BJ1 on iru had its highest growth concentration at 36 h, enter stationary phase between 36 to 60 h before an increase in growth at 72 h. Considering the optical density used in measuring the population of the isolates as the fermentation takes place it can be observed that when *B. subtilis* IU201 and *S. epidermidis* U259 were cultured together there was an increase in the population size and optimum growth achieved after 48 h of fermentation compared to when *B. subtilis* IU201 was used alone, but there was a significant reduction in population size when *L. plantarum* BJ1 was added. It was also

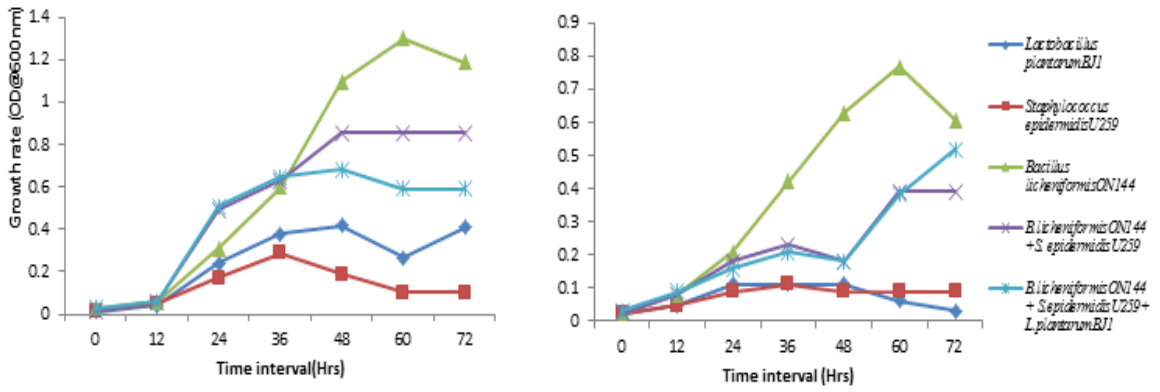

Figure 3. Growth rate of *Bacillus licheniformis, Staphylococcus epidermidis* and *Lactobacillus plantarum* in ”iru” and "ogiri egusi"

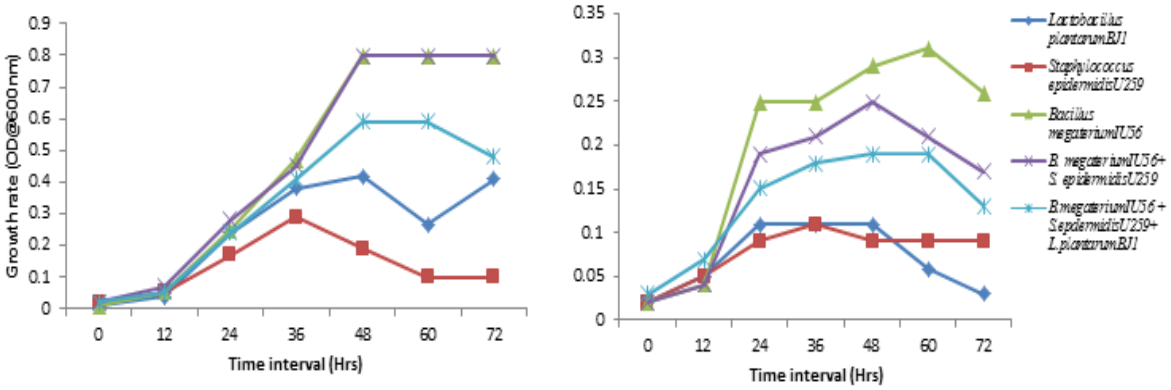

Figure 4. Growth rate of *Bacillus megaterium, Staphylococcus epidermidis* and *Lactobacillus plantarum* in “iru” and "ogiri egusi"

observed that the growth rate of *S. epidermidis* and *L. plantarum* when used singly is very minimal. For *B. pumilus* IU617 grown singly on iru, it had its highest growth concentration at 60 h. When cultured with *S. epidermidis* U259 highest growth was observed at 48 h and it enters the stationary phase between 60 to 72 h. When co cultured with *S. epidermidis* U259 and *L. plantarum* BJ1 it reached optimum growth at 36 h, enter stationary phase from 36 to 60 h with a later increase in growth at 72 h (Figure 2). The same pattern was observed for *B. licheniformis* ON144 but when cultured with the other isolates the stationary phase was between 36 to 48 h with a decrease in growth rate from 60 h (Figure 3). However, figure 4 represented the growth rate of *B. megaterium* IU56, when inoculated singly and in combination with *S. epidermidis* U259 had its highest growth at 48 h respectively, but when the three isolates were cultured together, the highest growth rate was recorded at 48 h, enters stationary phase at 48 to 60 h and it declines at 72 h.

For ogiri egusi (melon seed), the relationship of *B. subtilis* IU201 with *S. epidermidis* U259 and *L. plantarum* BJ1 was presented in Figure 1 and it was observed that highest growth rate was achieved at 72 h and entered stationary phase from 36 to 60 h, (Figure 1). For *B. licheniformis* ON144, when inoculated singly on ogiri egusi, after the logarithmic phase for 6 h there was an increase in growth rate reaching its peak at 60 h before declining at 72 h, combination of the three isolates leads to an initial increase in growth from 12 – 36 h, the growth decline at 48 h and later increase at 48 – 72 h, this is presented in Figure 2. Mixed cultures of *B. megaterium* with *S. epidermidis* and *L. plantarum* enter its stationary phase from 36 to 60 h before experiencing a decrease in growth at 72 h while *B. pumilus* with other isolates experienced stationary phase from 24 to 48 h followed by an increase in growth at 60 h (Figure 3 and 4). In all the fermentation processes, single culture of S. epidermidis and *L. plantarum* had the least growth rate for both Iru and Ogiri egusi.

Discussion

Fermentation of vegetable protein is usually by chance inoculation brought about by various species of microorganisms which are also an integral part of the processing system and are usually present in succession. Smid and Lacroix 2013 reported

that most food fermentation processes depend on mixtures of microbes which act in concert to produce the desired product characteristics, members of the consortium communicate with one another by trading metabolites or by exchanging molecular signals. As a result each individual cell in the mixture responds to the presence of others in the consortium and they also carry out division of labour between the members of the consortium leading to an overall output that can only be explained by combining tasks performed by constituent individuals or subpopulations (Ayad *et al.*, 2001).

Two types of condiments used in this study, iru and ogiri egusi, their pH range from 5.8 – 8.3 after fermentation. Ammonia is released and the pH rapidly reaches as high as 8.0 or higher. The combination of high pH and free ammonia along with very rapid growth of the essential microorganisms at relatively high temperatures above 40°C make it very difficult for other microorganisms that might spoil the product to grow. Thus, the products are quite stable and well-preserved especially when dried. Increase in pH into the alkaline range may be physiologically important for tolerance and adaptation of fermenting microorganisms in the environment. Increase in pH due to the formation of ammonia from amino acids during fermentation may consequently encourage growth of spoilage organisms (Achi, 2005).

A total number of 168 isolates were characterised and identified with *Bacillus* spp having the highest frequency of occurrence (72%). Members of the genus *Bacillus* have consistently been reported to be responsible for the fermentation of some vegetable proteins in West Africa especially iru and ogiri egusi (Odunfa, 1981; Odunfa and Oyewole, 1986; Azokpota *et al*., 2006; Enujiugha, 2009; Osho *et al*., 2010). Bacilli have proteolytic ability and are able to degrade oil and the proteins are hydrolysed to peptides and amino acids. *Bacillus* sp were the most predominant in the fermentation process and this is in agreement with the observation of Beaumont that *Bacillus* sp constitute over 95% of the total microbial population density in iru and ogiri fermentation because *Bacillus* cells exhibit very high protease activity compared with the other bacterial isolates.

The dominance natures of *Bacillus* spp suggest that they could be targeted as possible starters for the manufacture of safe and reproducible quality products (Odunfa, 1981; Osho *et al*., 2010). Furthermore, species of B. subtilis group have been reported to be generally regarded as safe (GRAS) by the U.S. Food and Drug Administration and their role in the fermentation of locust bean has been thoroughly investigated (Prabir *et al*., 2014). *Staphylococcus* sp. found cannot be ascertained to play a significant role in the fermentation processes because non fermenting species may just be ubiquitous contaminants although they may affect the flavour of the final product when occurring in high numbers (Steinkraus, 1995). Lactic acid bacteria are generally fastidious on artificial media, but they grow readily in most food substrates and lower the pH rapidly to a point where competing organisms are no longer able to grow (Daeschel *et al*., 1987). The low growth rate of *Lactobacillus plantarum* in this study for both fermented condiments used signifies that the organism was not able to lower the pH of the condiments and therefore could not suppress the growth of *B. subtilis* and *S. epidermidis* which invariably affects its growth rate. Nevertheless, lactic acid fermentations have other distinct advantages in that the foods become resistant to microbial spoilage and toxin development.

Three different isolates were co - cultured for the fermentation of iru and ogiri. Microbial interaction between *Bacillus subtilis* and *Staphylococcus epidermidis* in iru gave an increase in population in relation with the optical density compared to when *B. subtilis* was used singly and when *Lactobacillus plantarum* was added to the culture. The dynamics of fermentation in any food matrix is a complex microbiological process involving interactions between different microorganisms (Bull and Slater, 1982), because microbial interactions in mixed cultures occur via multiple mechanisms (Smid and Lacroix, 2013). Such interactions may be direct, such as through physical contact, or via signalling molecules (Keller and Surette, 2006). Alternatively, indirect interactions may occur where changes in the physicochemical properties of the environment induced by one strain trigger a response in another strain (Fredrickson, 1977; Hugenholtz, 1986) and the effects of such interactions on the fitness of the strains involved may either be positive, neutral, or negative. Co – culturing *B. subtilis* and *S. epidermidis* showed that a stable co – existence occurred between the two microbes signifying a mutualistic mode of interaction. It was established that both interacting strains gain fitness. The mutual effects on fitness of interacting strains are an effective means of classifying interactions (Benkerroum *et al.,* 2005). In this study, when three isolates are co cultured together for the fermentation process it was observed that rate of growth of the organisms reduced compared to when only two isolates were cultured together. The possible explanation is that the type of interaction between these isolates was that of amensalism in which one organism adversely affects the other organism without being affected

itself. This pattern of interaction frequently occurs in food fermentations since the major end products of primary metabolism such as carboxylic acids and alcohols are effective growth inhibitors of indigenous microbiota. Moreover, production of antimicrobial compounds, such as bacteriocins, that are produced by many food-fermenting lactic acid bacteria plays an important role in mixed-culture population dynamics and this explains why there is a reduction in growth rate of the isolates when *L. plantarum* was added. Typically, bacteriocin-producing strains produce a dedicated immunity system that protects the host from detrimental effects (Stecchini *et al*., 1991; Malakar *et al*., 1999, 2003; Leroy *et al*., 2007). The production of bacteriocins by these lactic acid bacteria is thought to support domination of the producer in the microbial community (competitive exclusion) (Leroy *et al*., 2007; Settanni and Corsetti, 2008). In terms of ecological typing, this can be considered as a form of amensalism. On the other hand, the same selecting principle also leads to stable co-existence between the bacteriocin producers and bacteriocin-insensitive microbes. This, however, does not lead to full dominance of the producer strain since it also faces competition for nutrients with insensitive microbes present in the culture. Moreover, the bacteriocins producer exerts a negative effect, if any, on a minor part of the microbial community and therefore does not experience any growth benefit of the eradication of its target. This demonstrates the complex web of interactions which exists in mixed cultures and this result because of cross-feeding and exchange of metabolites among the community (Settani and Corsetti, 2008). Coexistence of *B. subtilis* and *S. epidermidis* for the fermentation of condiments is that of a mutual effect with both participating microorganisms deriving a benefit from the interaction.

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