academicJournals

Vol. 16(2), pp. 58-64, 11 January 2017
DOI: 10.5897/AJB2016.15714
Article Number: 5C36C5162374
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antibacterial potential components of *Bacillus* species and antibiotics residues in branded and unbranded honey samples from Nigeria

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Received 5 October, 2016; Accepted 23 December, 2016

Honey is a sweet viscous liquid produced by honey bee, *Apis mellifera* from the nectar of plants. Honey is a natural product that has been used from ancient times till now as food and for medicinal purpose. This study was carried out to determine the mode of action of *Bacillus* species and antibiotics residues in branded and unbranded honey samples from Nigeria. *Bacilli* spp. count was carried out by initially heating diluted honey samples in water bath set at 80°C for 15 min, while total bacterial count was carried out using the pour plate method. Antibacterial activity of identified *Bacillus spp on Micrococcus* was determined using well-in agar method while the mode of action was carried out by reporter assay method. Detection of tetracycline, gentamycin and streptomycin was analyzed using high performance liquid chromatography (HPLC) column oven L-2300 and Column intensil ODS-3C18 (250 x 4.6 mm). Honey samples (2 g) were extracted for HPLC by deprotenizing using acetonitrile and methanol with flow rate of 1 mL/min and RID detector was used to detect antibiotic residue. *Bacilli* from honey were characterized physiologically, morphologically and biochemically, they were tentatively identified as *Bacilli licheniformis, Bacilli subtilis, Bacilli coagulans, Bacilli cirulans, Bacilli pumilis* and *Bacilli badius*. The most prevalent *Bacillus* spp. were *B. licheniformis* and *B. subtilis.* Total bacteria count for branded honey ranged from 2.2 x 10^2 to 5.5 x 10^3 cfu/g, while *Bacilli* count ranged from nil to 6.2 x 10^2 cfu/g. For unbranded honey samples, total bacteria count ranged from 7.0 x 10^3 to 3.5 x 10^2 cfu/g, while *Bacilli* count ranged from 5 x 10^1 to 1.6 x 10^3 cfu/g. Four of the isolates representing branded (SF2 and RW2) and unbranded honey samples (EH2 and TC2) exhibited antibacterial activity against *Micrococcus*; one isolate (SF2) showed cell wall causing antibacterial activity. Tetracycline was detected more in the unbranded honey samples while gentamycin and streptomycin were detected in just two unbranded honey samples, indicating that tetracycline is used frequently for the treatment of bee diseases that is why it is detected as residue in the finished honey product.

Key words: Antibiotics, *Bacillus*, health benefit honey, high performance liquid chromatography (HPLC), residues.

INTRODUCTION

Honey is a sweet viscous liquid produced by honey bee, *Apis mellifera* from the nectar of plants (EC, 2001).

Honey is made up of starch: 1%, triaccharides: 2%, maltose: 7%, water: 17%, glucose: 31% and fructose: 38% (Vanhanen et al., 2011; National Honey Board, 2008). Honey consumption in developing countries such as China, Argentina, India, Brazil and Egypt is estimated to be 0.1 to 0.2 kg per capita. Developed countries consume generally higher amounts (Bogdanov, 2009). Product of honey fermentation are honey vinegar, honey beer and alcoholic beverages. It is also used for cosmetic products such as lotions, ointments, creams, perfumes and so on. The quality of honey produced in recent years have decreased, this might be due to infection of bees by microorganisms, resulting in the death of bees and also the quest for wealth has led to the use of antibiotics to increase the yield of honey production. Others add sweeteners to increase the volume of honey, thus reducing the quality and its antimicrobial capacity (Crosby and Alfred, 2004). Branded honey is processed and well packaged, they usually have a brand name and somestimes NAFDAC number as in the case of Nigeria. Some branded honey might have been analysed by been exposed to high level of heat, pollens are often filtered off and pasteurized thus making the product appealing to the eyes. Most of them lack some basic health benefits and the origin cannot be detected. Unbranded honey do not have brand names and they are usually the local products. They are raw honey from the farm harvested by local farmers who may or may not be into bee farming. The handling of unbranded honey is usually poor and does not look presentable but have heath benefits.

The extensive use of antibiotics in beekeeping has led to an accumulation of antibiotic residues in honey, thereby leading to decreased quality and difficulty in marketing (Tillotson et al., 2006; Sapna and Nimisha 2010; Orianne et al., 2013; Reybroeck et al., 2012; Mahmoudi et al., 2014). Antibiotics are used in beekeeping as growth promoters and to treat diseases. Research has shown the presence of antibiotics residue in honey. For example, oxytetracycline and chloramphenicol residues have been found to be above the regulatory standards in honey (Saridaki-Papakonstadinou et al., 2006; Ortelli et al., 2004); other antibiotics such as erythromycin, lincomycin, monensin, streptomycin and enrofloxacin have also been detected in honey (Imad et al., 2013). Antibiotic residues have a relatively long half-life and may have direct toxic effects on consumers and also result in drug resistant organisms. The microbes found in honey and honeycomb are bacteria, molds and yeast; they come from the bees, nectar or from external sources. Honey composition has an effect on the growth and survival of many species of microorganisms by bacteriostatic or bactericidal, based on this, it is expected that honey should contain small number and limited variety of microorganisms (Snowdon and Oliver, 1996; Snowdon, 1999; Olaitan et al., 2007). Studies carried out has shown that the intestine of bees contains 1% yeast, 27% Gram-positive bacteria (*Bacillus, Bacteridium, Streptococcus* and *Clostridium* spp.) and 70% Gram-negative bacteria (*Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus* and *Pseudomonas*).

Few pathogens have been found in honey (Snowdon and Oliver, 1996, Snowdon, 1999; Evans and Armstrong, 2006). *Bacillus* spp. are the most predominant microorganism in honey; they possess spores and have the ability to withstand the characteristic nature of honey (Nevas et al., 2006). A study carried out by Esawy et al. (2011, 2012) reported a novel *Bacillus subtilis* in honey isolate as a new source of very important enzymes such as levansucrase, dextranase and lipase. *Bacillus* strain Mori 2 has been isolated from honey. It has the ability to synthesize surfactin and also has anti-*Paenibacillus larvae* activity. *B. subtilis* Subsp. *Subtilis* Mori 2 was isolated from honey sample in Morillos Argentina (Sabate et al., 2012). The present study aimed to dentify the predominant *Bacilli* spp. in branded and unbranded honey samples from Nigeria and their ability to produce components that can inhibit pathogenic organisms. The study also investigated the presence of antibiotics residue in the selected honey samples in order to advice if the components can be used in bee farming rather than antibiotics because honey is consumed.

MATERIALS AND METHODS

Honey samples

Eighteen honey samples obtained from Nigeria were used for the study. Seven of them were branded honey obtained from supermarket in Rivers State, Nigeria and the remaining 11 were unbranded which were obtained from the local retailers at various locations in Nigeria.

Enumeration and identification of Bacilli spp.

Ten grams of each honey sample was weighed and diluted in 90 ml of sterile saline water. Two (2) milliliters of diluted honey samples were transferred in duplicates into sterile test tubes. One set of the tubes was heated in water bath set at 80°C for 15 min for *Bacilli* count. The second set of tubes was not heated for total count. Ten-fold dilutions was carried out and 1 mL of 10^2 – 10^6 dilutions were prepared and used for pour plate on Nutrient Agar (HiMedia). Plates were incubated aerobically at 37°C for 24 to 48 h. Morphological characteristics such as the shape, colour and elevation were examined. The number of colony forming unit (cfu/g) was recorded by counting the number of colonies on each plate ranging from 30 to 300. Representative dorminant colonies were isolated and purified by streaking several times on Nutrient Agar.

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Stock cultures were maintained in slants for further study, isolates were identified using the Bergey's Manual.

Taxonomic identity

Gram staining of 24 h grown culture (16 ± 2 h at 37°C) of *Bacillus* isolates was carried using standard procedure (Cappuccino and Sherman, 2002). Prepared cultures were examined by phase contrast microscopy (DP50 digital camera, Olympus Optical Co. Ltd., Tokyo, Japan). Scanning electron microscopy (SEM) was carried to further identify the cell morphology and isolates purity. Freshly grown isolates were harvested and fixed by 0.3% glutaldehyde, dehydrated and embedded using the method described by McDougall et al. (1994). Motility was also carried out using semi-solid agar medium in a test tune. The isolates were inoculated with a straight wire making a single stab down the center of the tube to about half the depth of the medium and incubated at 37°C.

Catalase test was carried out using a loopful of overnight grown culture. Cultures were smeared on a clean glass slide and 3% H_2O_2 was added and allowed to react. The presence of effervescence was recorded as catalase positive (Vanderzant ,1992). Starch hydrolysis was carried out by streaking overnight culture on starch agar plates (1% starch in NA) and incubated at 37°C for 24 to 48 h. After incubation, plates were flooded with iodine solution for 15 to 3 min and examined for the formation of clear zone.

Gelatin hydrolysis, lecithin test, casein hydrolysis and hemolytic effect were carried out using the the methods of Sorokulova et al. (2008). Voges-Proskauer (VP) test on *Bacillus* isolates was carried out using the methods of Cappuccino and Sherman (2002).

Physiological tests on the *Bacillus* isolates were carried out on LB broth with different pH (2.0, 4.0, 6.5, 8.0 and 10.0) which was prepared by adjusting the medium by using 0.1 N NaOH/HCl (Cappuccino and Sherman, 2002). After sterilization by autoclaving, the aliquots of sterile LB broth (5 ml) with different pH values were inoculated with 50 µL (10^5 to 10^6 CFU/ml) of freshly grown (16 ± 2 h) *Bacillus* isolates, this was ensured by using Mc Farland Standard. The aliquots were then incubated at 37°C for 24 to 48 h and observed for growth which was indicated by turbidity change.

Growth at different temperature was carried out, LB broth (5 ml) were inoculated with 50 µL (10^5 to 10^6 CFU/ml) of freshly grown *Bacillus* isolates and incubated at different temperatures (4, 15, 25, 35, 45 50 and 55°C) for 24 to 48 h. After incubation, the tubes were observed for growth by turbidity change. Growth at different salt concentrations was carried out on LB broth tubes prepared with different salt (NaCl) concentrations (4, 5, 6.5 and 8%) were inoculated with 50 µL (10^5 to 10^6 CFU/ml) of freshly grown *Bacillus* isolates and incubated at 37°C for 24 to 48 h. After incubation, growth observation was made based on the turbidity change using Mc Farland standard.

Enzyme and antibiotics assay

Alpha-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure described by Bernfeld (1955) using 1% soluble starch. One milliliter of the enzyme was incubated for 15 min at 55°C with 1 mL of 1% soluble starch in 0.1 M phosphate buffer. The reaction was stopped by addition of 1 mL DNS and heated in boiling water for 10 min and allowed to cool in running tap for 5 min. Absorbance was read at 540 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 µmole glucose equivalent per minute under the assay conditions.

Antibacterial activity and detection of mode of action (MOA)

The inhibitory activity of the *Bacillus* isolates was determined using well diffusion method of 18 – 24 h neutralized cell-free supernatant on indicator strain *Micrococcus luteus* ATCC 9341 (Devi and Halami, 2011). Cell wall stress response reporter strain *B. subtilis* BSF 2470 for lipid II interfering antibiotics was used in this study. Chromogenic plate assay as described by Burkard et al. (2007) and Mascher et al. (2008) was followed for detecting MOA of the *Bacillus* isolates. A vertical streak of reporter strain was made on LB agar plates supplemented with X-Gal, and then the *Bacillus* isolates were streaked horizontal to the vertical line. The plates were incubated for 24 to 36 h at 37°C and blue coloration of the reporter strain was observed at the inter-junction, which indicates the inhibitory MOA of the tested culture on cell wall. The culture, *B. subtilis* ATCC 6633 was used as a positive control and a negative control was also used.

Estimation of antibiotics by HPLC

Honey samples were extracted by deproteinizing chemical procedure using acetonitrile (ACN). Two grams (2 g) of honey sample was placed into 10 mL test tube and shaken intensively with 3 mL ACN for 1 min. The mixture was centrifuged for 15 min at 5000 rpm. Supernatant was collected and dried under nitrogen gas. The residue was re-dissolved in 3 mL of methanol, filtered through 0.45 µm and 10 µl was injected into HPLC system. The determination of antibiotic residue in honey samples was carried out according to the method of Shafqat et al. (2012), Ashad et al. (2012), Imdad et al. (2013). HPLC (Schimad Zu Japan) was used to detect the presence of tetracycline, streptomycin and erythromycin in the samples. Antibiotics were obtained from Hi-Media (India). Separation was achieved using Column oven L- 2300 and column Intersil ODS -3 C18 (250 x 4.6 mm). Dimension of stationary phase was 5 µ, pump range accuracy of flow was 1 ml/min and RID detector was used in the detection of antibiotic residue. All the solvents were filtered through 0.45 µm membrane filter. Antibiotics detection was performed using different mixture of an aqueous mobile phase A (60%) and acidified water and organic mobile phase B (40%), methanol and ACN with flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Enumeration of total microbial and Bacilli count

Total count and total *Bacilli* counts was carried out in branded and unbranded honey samples, total count for branded honey ranged from 2.2×10^2 to 5.5×10^3 cfu/g, while *Bacilli* count ranged from nil to 6.2×10^2 cfu/g. Total count for unbranded honey samples ranged from 3.5×10^2 to 7.0×10^3, cfu/g while *Bacilli* count ranged from 5×10^1 to 1.6×10^3 cfu/g (Table 1). According to the Resolution MERCOSUL GMC, number 15/94 technical rules for identity fixation and honey quality approved by ordinance number 367 from September 4th 1997, honey may contain a maximum number of 100 cfu/g. The results obtained from total and *Bacilli* count indicate the presence of outstanding microbial load in the unbranded honey samples which could be attributed to the unprocessed nature of the product. *Bacilli* count was found in most of the honey samples except for one (LP) unbranded honey. The presence of *Bacillus* in honey shows that they can survive in honey in dormant state; hence they are sometimes called friendly bacteria

Table 1. Total mesophilic bacterial count, total *Bacilli* count and antibiotics detected in honey samples.

Honey sample	Sample code	Total count (cfu/g)	Total bacilli count (cfu/g)	Antibiotics detected (µl/ml)
Udi	UD	3.5×10^2	1.5×10^2	Not detected
Ogboloma	OG	6.0×10^3	1.0×10^3	Gent(6.11)
Foreign Honey 1 (SM)	FH	3.6×10^2	1.2×10^2	Tet (6.20)
Honey farm (Rivers)	SF	3.0×10^3	1.0×10^2	Nil
Shadam (Jos)	SJ	4.5×10^2	3.2×10^2	Tet (3.41), (Gent 4.3)
Foreign Honey 2	PH	5.0×10^2	1.5×10^2	Tet (4.0)
Etche	EH	3.9×10^2	5×10^1	Tet (8.562)
Agbor (Delta)	AG	4.0×10^3	6.2×10^2	Tet (1.47)
Foreign Honey 3 (SM)	RW	5.5×10^3	4.6×10^3	Strep(1.68), Tet absent
Obudu (Cross River)	OD	6.5×10^2	4.2×10^2	Tet(1.2)
Taraba (wild honey)	TW	4.9×10^3	7.8×10^2	Not detected
Lagos	LA	6.4×10^3	5.5×10^2	Not detected
Ezikie (Enugu)	EZ	7.0×10^3	1.6×10^3	Not detected
Taraba	TC	3.8×10^3	1.6×10^3	Not detected
PG natural Honey	PG	6.2×10^2	4.9×10^2	Not detected
Umudike	RC	7.5×10^2	5.2×10^2	Gent (7.74)
Foreign Honey 4	LP	2.2×10^2	Nil	Tet absent
Petro Honey	PR	8.5×10^2	6.1×10^2	Strep (2.55)

Tet: Tetracycline, Gent: Gentamycin, Strep: Streptomycin.

because they may not necessarily be harmful.

Taxonomic identification of *Bacillus* spp.

Twenty-six isolates were selected from the 18 honey samples for further identification. All the isolates selected were found to be Gram-positive rod shape. Twenty-four of the isolates were catalase positive and negative for RW3 and SJ3. Most of the isolates were motile except RW1, RW2 and EH2. Isolates were confirmed as *Bacillus* spp. by their heat tolerance, Gram staining, catalase reaction and other biochemical test were carried out.

Electron microscopic studies of the selected *Bacillus* isolates showed differences in cell morphology and cell width (Plate 1A and B). The rod shape and size also varied in the different isolates. Twenty-six *Bacillus* isolates were cultured in LB broth at pH 2, 4, 6.5 and 10. Most of the isolates were able to grow at pH 8 and 10, while none of the 26 isolates grew at pH 4. Isolate AD1, TW2, TC1 and TC2 did not grow in pH 2. The range of temperature growth was between 30 and 45°C, while isolates UD1, SJ2, EH2, RW1, RW2 and TC1 & 2 grew at 50°C. Most of the isolates did not grow at temperature 55°C except isolate UD1, UD2, SJ2, TW1 and TW2.

Antibacterial activity and mode of action

Identified *Bacillus* spp. were screened for antibacterial activity against the indicator, *Micrococcus luteus* ATCC 9341. Four out of 26 isolates showed inhibitory effect against *Micrococcus luteus* ATCC 9341 (Plate 1C and D). The four isolates were further screened to know their mode of action (MOA). The antibacterial substances produced by the different *Bacillus* isolated from honey samples differed in their MOA. The reporter strain that effectively detected MOA of antibacterial substances of *B. subtilis* BSF 2470 was used to sense the lipid 11 interfering antibiotics via RS two component response regulator system which results in change in colour (Mascher et al., 2004; Devi and Halami, 2001). When subjected to MOA, isolate SF2 is on the cell wall (Plate 1). Isolate EH1, RW2 and TW1 antibacterial activity may be due to some other molecules that the bacteria exported. Thus, *Bacillus* spp. from honey samples which showed antibacterial activity could be used to treat bee diseases instead of use of antibiotics. This shows that some *Bacillus* in honey are friendly, thus the therapeutic properties of honey. The current ban of the use of antibiotics as therapeutic agents against bee infections has led to research for natural alternatives. The components produced by these *Bacillus* spp. from honey and bees can be further researched on and applied in science (Alberori et al., 2016). The bioactive metabolites of these species can be characterized to determine their mechanism of action behind their antibacterial activity and therapeutic characteristics. A study carried out by Olofsson et al. (2014) showed that LAB symbionts are source of unknown factors that contribute to honey properties and they were able to inhibit wound pathogens such as MRSA and VRE among others. Their study

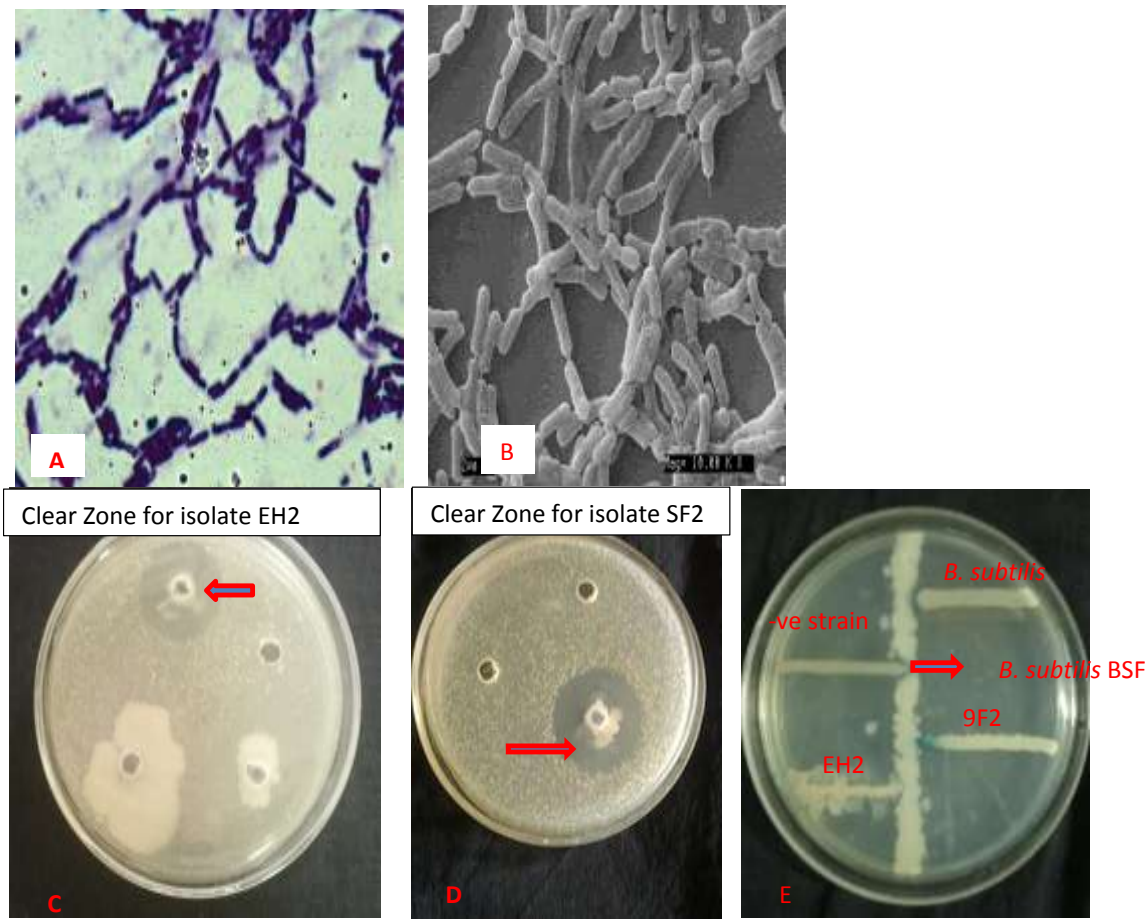

Plate 1. (a) Gram reaction; (b) SEM; (c and d) antibacterial activity of *Bacillus* sp. against *Micrococcus luteus* ATCC 9341; (d) chromogenic assay of *Bacillus* spp.

confirmed that honey contained myraid active compounds that can be applied against human infections due to resistant microorganisms.

Enzyme assay

Results obtained from starch hydrolysis showed that 90% of the isolates could hydrolyze starch except SJ1, 2, 3 and PR1 & PR2. Hemolytic activity was seen in most of the isolates which suggests that they are pathogenic strains except for isolate UD2, SF 1, 2, 3, TW2 and RF1. Isolates UD1, 2, 3, OG1, 2, SF1, 2, 3 and RC1 were unable to hydrolyze phospholipids on egg yolk plates, thus they can be considered nonpathogenic. The isolates were identifined using the methods Bergy's Manual of Systematic Bacteriology (Sneath et al., 1959). The isolates from honey samples were tentatively identified as *B. licheniformis, B. subtilis, B. coagulans, B. cirulan* and *B. pumilis.* Sherwani et al. (2013) identified *B. polymyxa, B. pumilis, B. cereus* and *B. megaterium.* Out of the 26 *Bacilli* isolates, 15 were able to produce alpha amylase enzyme. One unit (U/mL) of α-amylase is defined as the amount of protein (α-amylase) required to liberate 1 µ mole (0.27 mg equivalence) of reducing sugar (D-glucose) from starch/min under the assay condition. Amylase ranged from 1.803 to 4.104 µ/mole (Table 2). The production of amylase by *Bacillus* strains from honey samples shows that *Bacillus* from honey could be new sources of very important enzymes that could be used in the food industry. Recent studies confirmed that *Bacillus* spp. from honey can be used for production of important enzymes such as levansucrase and other important enzymes (Esawy et al., 2011, 2012; Safty, 2011).

Antibiotics detection

The branded and unbranded honey samples were evaluated for the presence of tetracycline, gentamycin and streptomycin. The detection of tetracycline in the honey samples was found to be among the highest and most frequent in occurrence than all the other antibiotics tested as shown in Table 1. This could be attributed to

Table 2. Taxonomic identity of *Bacilli* isolate and alpha amylase production.

Isolate	Source of honey	Tentative identity	α-Amylase (µ/mole)
UD1	Udi (unbranded) NIG	*B. licheniformis*	2.344
UD2	Udi (unbranded) NIG	*B. licheniformis*	Absent
OG1	Ogboloma(unbranded) NIG	*B. subtilis*	2.537
OG2	,,	*B. badius*	Absent
SF1	Honey farm (branded) NIG	*B. circulans*	2.184
SF2	,,	*B. subtilis*	2.598
SJ1	Shadam Jos (unbranded) Nig	*B. badius*	Absent
SJ2	,,	*B. badius*	Absent
SJ3	,,	*B circulans*	Absent
PH1	Foreign honey 2 (branded)Hongkong	*B. licheniformis*	Absent
PH2	,,	*B. subtilis*	Absent
EH1	Etche (unbranded) Nig	*B. licheniformis*	3.492
EH2	,,	*B. subtilis*	3.160
EH3	,,	*B. subtilis*	2.733
AD1	,,	*B. subtilis*	2.184
RW1	Foreign honey 3 (unbranded) CHINA	*B. subtilis*	4.104
RW2	,,	*B. coagulans*	3.202
RW3	‘’		2.128
TW1	Taraba wild (unbranded) Nig	*B. licheniformis*	2.709
TW2	,,	*B. circulans*	2.254
TC1	Taraba comm. (unbranded)	*B. licheniformis*	2.051
TC2	,,		1.803
RT1	Umudike (unbranded) Nig	*B. pumilis*	Absent
RT2	,,		Absent
PR1	Petro honey (unbranded) Nig	*B. licheniformis*	Absent
PR2	,,	*B. pumilis*	Absent

the fact that tetracycline is frequently used based on its effectiveness in beekeeping. Tetracycline was detected in both branded and unbranded honey samples but more in unbranded honey. The presence of gentamycin and streptomycin was seen in only two unbranded honey samples, these antibiotics were not detected in any branded sample. The presence of gentamycin in two honey samples is contrary to the findings of Imdad et al. (2013) where gentamycin was not detected in all the honey samples tested. The presence of antibiotics in honey has become alarming due to the broad use of antibiotics for the treatment of bee diseases. There is ban currently in some countires on the use of antibiotics to treat bee infections in honey production; the presence of these residues in honey after being consumed for a long time can cause health challenge. It is necessary to check the importation of honey into Nigeria. The US, some years ago, banned the importation of honey from China and Asian countries into US due to the presence of illegal animal antibiotics, sweetners used; they were mislabeled, and heavy metals were found in those honey. Animal antibiotics found in honey such as chloranphenicol can cause cacinogenicity, serious fetal reaction, aplasic anemia and other health related issues (Shafqat et al., 2012).

Conclusion

Bacillus spp. are prevalent in honey samples due to the microbial flora of the honey bee themselves, pollen or external sources. This study revealed that certain *Bacillus* spp. from honey contains certain antimicrobial components that can be used to inhibit the growth of pathogenic organisms such as *Micrococcus letus.* Further studies can be carried out on this aspect of the work to determine what these components are. The *Bacillus* spp. also exhibited the production of enzymes so they can be novel sources of bioactive compounds that could be used in apiculture. However, the presence of antibiotics residue in honey beyond the maximum residual limit is of great concern since that could pose serious health challange and also increase drug resitantance. Honey imported into the country should be properly screened to know the source and its quality. It is advisable that the stipulated guidelines by WHO and individual countries for honey production and antibiotics usage in beekeeping should be adhered strictly. Honey should be produced

without destroying the beneficial microorganisms in it.

Conflict of interests

The authors have not declared any conflict of interests.

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