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Preliminary Detection of *Bacillus* species in Commercial Honey

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***Authors'* contributions**

This work was carried out in collaboration between all authors. Author OEA designed the study, wrote the protocol, carried out the research work and wrote the first draft of the manuscript. Authors TVO and NFP supervised, managed the literature searches and corrected the work. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: This research is aimed at detecting the presence of *Bacillus* species in honey using morphological characteristics, biochemical tests and preliminary molecular studies.
Place and Duration of Study: The study was carried out in the department of Molecular Biology, Institute of Medical Research Yaba Lagos, Nigeria. The study was carried out from June to July 2012.
Methodology: A total of 33 honey samples were used for this study, twenty- eight of the honey samples were of local origin while 5 were of international origin. Twenty-eight commercial honey samples were obtained from the six geographical regions in Nigeria from commercial retailers. The five foreign honey samples were obtained from the supermarkets namely: Friz fruit, Blossom, Forever, Aloe Vera and Rose honey, all of international origin. The honey samples were inoculated into sterile agar, blood and tryptone soy plates using the spread plate technique. Isolates obtained were purified and subjected to morphological tests, biochemical tests and further identification using polymerase chain reaction.
Results: All the honey samples had microbial growth in them, higher counts were observed in the commercial honeys from retailers than the foreign honey samples. Forty isolates suspected to be *Bacillus* from biochemical tests were subjected to PCR, 14 from the 40 were confirmed to be *Bacillus* spp.

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Conclusion: Microorganisms in honey cannot be identified fully with morphological and biochemical examinations alone, but combine use of morphological, biochemical tests and PCR technique is more accurate and reliable method of identification.

Keywords: Antibacterial; Bacillus species; biochemical test; honey; microorganisms; polymerase Chain Reaction.

1. INTRODUCTION

Honey is a natural sweet substance produced by *Apis mellifera* bees from the nectar of plants [1]. Honey is currently used in most culture for the healing of wounds, burns, rashes and ulcers. This information has led current scientists to study the properties and the therapeutic effects of honey, these studies have confirmed the use of honey for medicinal purposes due to its natural components. The components found in honey include high acidity, hydrogen peroxide, minerals, antioxidant, antibacterial activity etc. All this components found in honey led scientists to focus their attention on the differences, benefits and effects found among honeys from different regions [2].Honey possesses antibacterial activity against Gram positive and negative organisms thus reducing the number of organisms that can grow in honey. Studies have shown that raw and commercial honey possessed antibacterial activity against bacteria in varying degrees. The antibacterial activity of honey has been confirmed by several studies, which have shown that raw and commercial honey has the ability to inhibit the growth of *Staphylococcus aureus*, *Shigella* spp and *E. coli* [3]. The differences in the potency of honey samples can be attributed to various factors such as the time of harvest, the source of the honey, level of hydrogen peroxide in honey, moisture content etc [4,5]. Organisms found in honey come from the nectar, pollen, processing area, machines and containers that are not properly washed, while other organisms found in honey are from the bee [6,7,8]. Honey contains *Bacillus* which is aerobic spore-forming bacteria that are found in honey as spores and they survive in honey for long periods withstanding heat and other thermal application. The quality of honey is affected most times by microorganisms present in honey. The antibacterial activity of honey plays a role in reducing spore formers to the minimal population in honey. This is not guaranteed due to adulteration and mode of preparation of honey in some regions [9,10].

The use of honey as a wound dressing agent has been argued against because of the risk of it possibly causing botulism. Microorganisms that can survive in honey are those that withstand the concentrated sugar, acidity and antimicrobial characteristics of honey. *Bacillus* species are the most prevalent in honey, followed by Gram- variable plemorphic bacteria, mould and *actinomycetes*. Gram negative rods (*Enterobacteriaceae*) have been isolated from bee larva, there by having the possibility of been found in honey. Studies have shown the following Gram negative bacteria found in honey are *Achromobacter*, *Citrobacter Enterobacter*, *Erwinia*, *Eschercihia coli*, *Flavobacterium*, *Proteus* and *Pseudomonas* [11]. There is a risk of introducing spores into wounds if honey is used as a dressing without been certain of the quality and source of the honey. Honey researchers in Nigeria have studied the antimicrobial , antioxidant and other parameters of various honeys from different regions in Nigeria [12-15]. Scanty research has been done on molecular studies of the microorganisms found in honey from Nigeria. The genus *Bacillus* sp. is ubiquitous, endospore- forming Gram-positive bacteria. Their primary habitat is soil and due to resistant spores they can colonize different environments such as honey, food, animals, insects e.t.c [16,17]. *Bacillus* species has a wide distribution in nature, they are frequently isolated from plants, but they are also well adapted for growth in the intestinal tract of insects such as

bees and animals [17]. Molecular techniques have enabled researchers to identify microorganisms more concisly, molecular studies have shown that greater number of bacterial species in samples and specimens than culture results shows. The continous use of culture and molecular techniques for the identification of microorganisms is necessary to compare and contrast these newer to modern techniques of identification [17].

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 33 honey samples were used for the study, out of the 33 twenty-eight were commercial honey produced locally in various states in Nigeria. They were obtained from commercial honey retailers. Five processed commercial honey samples of foreign origin were purchased from the supermarkets (blossom, Aloe Vera, Forever, Rose and Frizfruit honey). Four honey samples were obtained from North- Central (Jos, Kogi), two from North – East (Jalingo), and two from North – West (Kaduna). Ten honey samples were obtained from South – East (National Root Crop Research Institute Umudike (NRCRI), Nsukka, Owerri). Eight honey samples were obtained from South- South (Warri, Yenegoa, Port Harcourt and Calabar) and two honey samples from South- West (Oyo) which were obtained from International Institute of Tropical Agriculture (IITA), Ibadan.

2.2 Isolation and Identification of Bacteria from Honey Samples

One hundred micro liters of honey samples was spread on nutrient agar plates (Oxoid), Blood Agar and Tryptone Soy Agar (Oxoid) plates. Plates were incubated for 24 hrs at 37ºC. The colonies on the plates were sub cultured onto nutrient agar plates to obtain pure colony. Pure colonies were stored and identified using standard microbiological and biochemical methods.

2.3 Determination of morphological characteristics of organisms

Morphological characteristics of the organisms were determined using the following test:

a. Gram Staining: This test was used to group the organisms into Gram positive and negative. It also differentiates them into rods or cocci. Bacilli are Gram positive rods that occur in chains [18,19].
b. Spore Staining: Spore staining technique was adopted to differentiate the spore forming rods (*Bacillus* spp) from the non- spore formers.
c. Motility test: The method of Barrow and Feltham was adopted to determine the motility of the isolates [18].

2.4 Biochemical tests

The following biochemical tests were carried out using the methods of Cheesbrough [19].

a. Sugar fermentation: this deals with the ability of different organisms to ferment glucose, sucrose, lactose, mannitol, fructose, maltose, xylose, raffinose and arabinose.
b. Oxidase test: this detects the presence of the enzyme cytochrome oxidase. The enzyme oxidizes the phenylenediamine in the reagent to a deep purple colour.

c. Catalase Test: This test detects the presence of the enzyme catalase. Catalase enzyme is found in most bacteria, it catalyzes the breakdown of hydrogen peroxide with the release of free oxygen. It differentiates catalase producing bacteria from non-catalase producers.
d. Citrate Utilization Test: this test is used in the identification of the enterobacteria. It is based on the ability of the organism to utilize citrate as its only source of carbon and ammonia as its only source of nitrogen.
e. Hydrogen Sulphide Test: This test is used to determine the ability of an organism to attack a specific carbohydrate incorporated into a basal growth medium with or without the production of gas, along with possible hydrogen sulphide.
f. Indole Test: this test is to detect the ability of an organism to breakdown tryptophan to pyruvate and indole. Most strains of *E. coli* and *Proteus* sp breakdown the amino acid tryptophan with the release of indole.
g. Methyl- Red Test: this is used to test the ability of an organism to produce and maintain stable acid and products from glucose fermentation.
h. Voges - Proskauer Test: It is used to detect the production of acetylmethyl carbinol (acetoin), a natural product from pyruvic acid in the course of glucose fermentation.

2.5 Primer Selection

The primer was selected through the combined use of four universal primers, XB4 employed as a 21–oligonucleotide reverse primer, 5'–GTG TGT ACA AGG CCC GGG AAC-3' and 5'-CAG ACT CCT ACG GGA GGC AGC AGT- 3', the primers amplify a 598 – base pair PCR amplicon. XB4 was located at base positions 1324-1304, in relation to *Stenotrophomonas maltophilia* (Gen Bank Accession number AY 169434). The primer used for this study was selected based on a previous study with slight modifications [20].

2.6 PCR Protocol Using Isolates from Honey

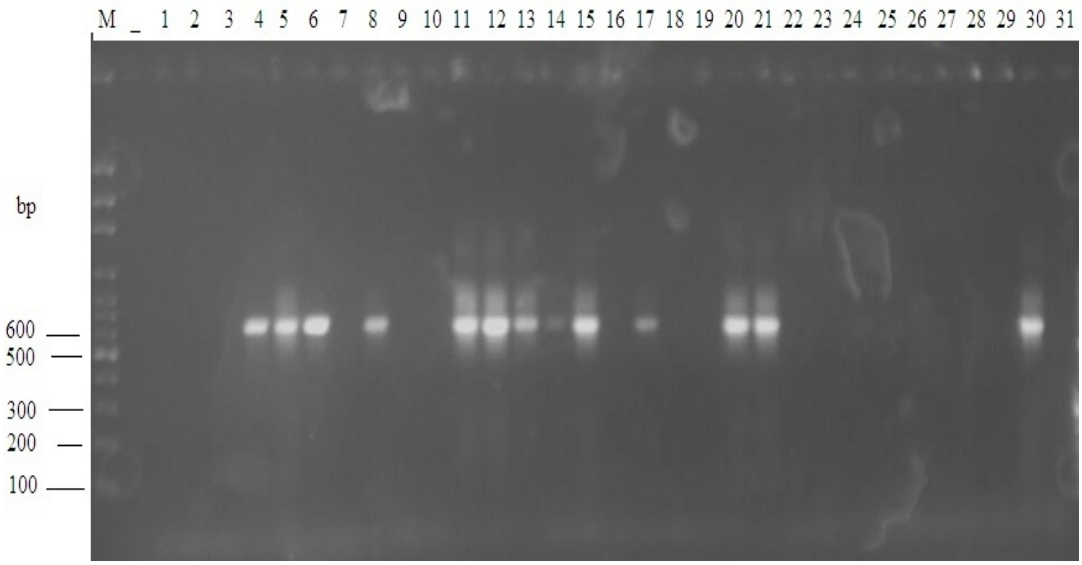
All isolates were sub cultured into Mueller Hinton Agar (Oxoid). Genomic DNA was extracted from a single colony with ZR Fungal/ Bacterial DNA MiniPrep^{TM}(ZYMO RESEARCH) purity kit. All the reaction mixes were set up in a PCR hood in a separate room from which the DNA was extracted and also the amplification and post-PCR room to minimize contamination. PCR was performed in 25µl of a reaction mixture containing DNA (10-200ng), 2µl of deoxynucleoside (d NTP) (Promega), 1.5mM $MgCl_2$, 1XPCR buffer, 1µl (each) of primers, 1 unit of Taq polymerase (Promega) and sterile distilled water. Following a hot start, the reaction mixtures were subjected to the following thermal cycling parameters in a Perkin Elmer 2400 thermo cycler: 96ºC for 3min followed by 40 cycles of 96ºC for 1 min, 55ºC for 1min, 72ºC for 1min, followed by 72ºC for ten minutes. The amplification product was separated on 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. One hundred (100) bp DNA ladder (Promega) was used as DNA molecular weight standard [21].

3. RESULTS AND DISCUSSION

The shapes of the colonies were examined on the plates after incubation periods of 24 hours at 37ºC. Most of the colonies appeared white, circular and flat after 24 hours incubation. Forty isolates from the honey samples were selected based on the morphological characteristics and features resembling *Bacillus* species. The isolates from the various honey samples were numbered from 1–40 and used for further identification. The morphological

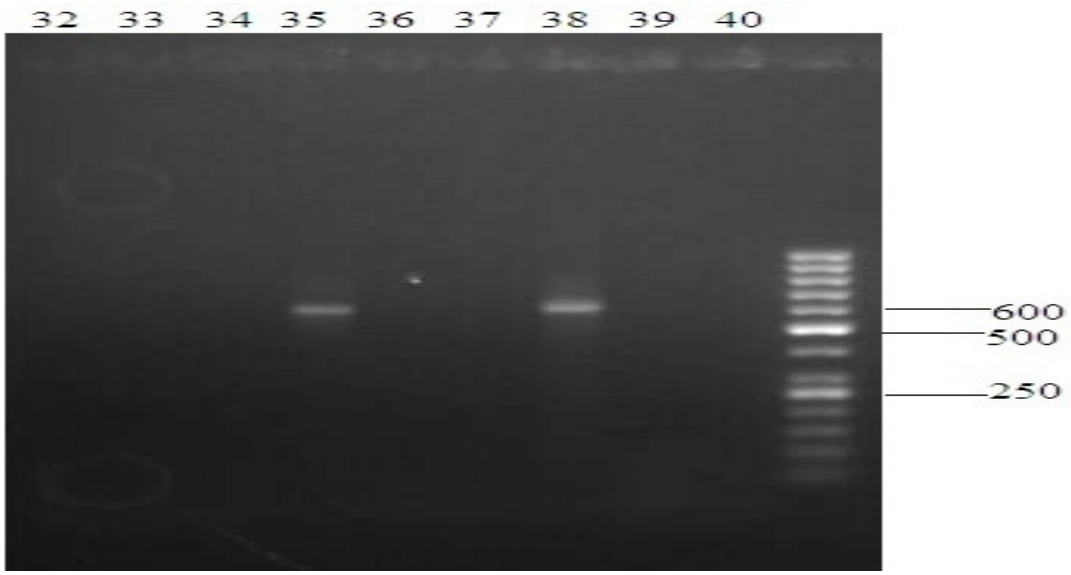
characteristics were confirmed after Gram staining using microscopic examination. The morphology of the isolates revealed that 80% of the isolates were gram positive rods, motile and possessed endospore. The isolates were opaque and wavy while isolate 7,22 and 23 were gram positive cocci as indicated in Table 1 below. Biochemical tests showed that the isolates were catalase positive and produced starch hydrolysis. Oxidase test and hydrogen sulphide fermentation were negative for all the organisms. The isolates were initially identified using morphological and biochemical tests. They were further subjected to PCR technique to confirm the identification. Eighty percent of these isolates had colonial apperence resembling *Bacillus* spp. Out of the forty isolates 3 were identified as *Micrococcus* sp. 2 were identified as *Eubacterium* sp. and rest 35 identified as *Bacillus* species using morphological and biochemical tests. The forty isolates were also subjected to PCR and 14 of them were identified as *Bacillus* spp as represented in Figs. 1 and 2. below. PCR amplification on high quality Genomic DNA preparations of these isolates generated amplicons for some of the isolates. The isolates that generated amplification are 4,5,6,8,11,12,13,15,17,20,21,30,35 and 38, these 14 isolates were confirmed as *Bacillus* spp using morphological, biochemical tests and PCR technique. This shows that the remaining 26 isolates were not *Bacillus* spp, they could be other organisms that can survive in honey such as *Clostridium* spp. They produced spores like *Bacillus* spp and they Gram positive. The 21 solateswere 1,2,3,7,910,14,16,18,19,22,23,24,25,26,27,28,29,31,32,33,34,36,37,39 and 40; as represented in Figs. 1 and 2. The results obtained from morphological and biochemical tests showed that these two methods of identification are not adequate to confirm the identification of an organism. Those identified as *Bacillus* species using morphological and biochemical tests were not confirmed when PCR technique was used, only 14 out of the 35 were confirmed.

In most institutions in developing countries, culture technique and biochemical tests are methods used for the identification of microorganisms from different sources. These methods are used because it is cheaper to afford and easy to come by. Culture technique and biochemical tests are time consuming, results obtained most times are not always accurate because isolates are prone to contamination and they are just preliminary methods of identification. In most cases reference isolates are not used during morphological and biochemical tests, thus affecting the results. The rapid advancement in molecular biology over the last 20 years has provided new techniques that are aimed at assisting in the detection and identification of micro-organisms from various sources. With the advent of molecular biology, microbiologists have another avenue for detection and identification of microorganisms. Using biolog method to isolate these organisms requires a complex media for growths which are expensive and are not available in some laboratories. DNA – based identification methods such as 16S rRNA gene sequencing and 16s- 23S intergenic region sequencing have been used widely for the purpose of identification and typing of microorganisms isolated from honey [22]. Unfortunately, no molecular identification has been carried out on the organisms isolated from Nigerian honey, this work was a preliminary study on the detection of *Bacillus* spp in Nigerian honey using morphological and biochemical tests which are frequently used in Nigeria and compared to PCR technique.



M=100bp

Fig. 1. Gel electrophoresis of the PCR amplified products for the detection of *Bacillus* species



100bp

Fig. 2. Gel electrophoresis of the PCR amplified products for the detection of *Bacillus* species

Table 1. Characterization and Identification of bacteria isolated from honey samples

S/NO	Isolate codes	Gram reaction	Catalase test	Methyl Red	Voges Proskauer	Indole production	Citrate test	Oxidase test	Motility test	Starch hydrolysis	Spore stain	Glucose	Lactose	Mannitol	Xylose	Galactose	Identified isolate
1	R1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
2	R2	+rod	-	+	+	-	+	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
3	R3	+rod	-	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
4	C1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
5	C2	+rod	+	+	+	-	-	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
6	C3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
7	C4	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Micrococcus sp*
8	N1	+rod	-	-	+	-	-	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
9	N2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
10	N3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
11	E1	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
12	E2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
13	E3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp*
14	I1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Eubacterium sp.*
15	I2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
16	FE1	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
17	FE2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
18	U1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
19	U2	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
20	U3	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
21	U4	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
22	F3	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Micrococcus sp.*
23	Ma	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Micrococcus sp.*
24	Mb	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
25	D1	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
26	D2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
27	K1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
28	K2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*

29	K3	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
30	IB1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
31	IB2	+cocci	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Eubacterium sp.*
32	B3	-rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
33	E1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
34	M1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
35	M2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
36	M3	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
37	BL1	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
38	BL2	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*
39	BL3	+rod	+	+	+	-	+	-	+	+	+	+	-	-	-	-	*Bacillus sp.*
40	BL4	+rod	+	+	+	-	-	+	+	+	+	+	-	-	-	-	*Bacillus sp.*

+ = Positive - = Negative

Bacillus species has a wide range of distribution in nature, they are frequently isolated from plants, and they have a well-adapted for growth in the intestinal tract of insects such as bees and animals [16]. Previous studies on honey identified 99% of *Bacillus* species by morphological examination and molecular biology analysis [7,23]. This is in conformity with the study carried out by another researcher that revealed the presence of microorganisms ranging from bacteria, moulds and yeasts in honey. Their study was carried out using morphological appearance of such isolate on specialize media [24]. Studies on honey have shown that honey produced by bees contains spore formers that come from the bees themselves, from pollens and other sources. The microbiological quality of honey in retail outlets and open markets should be properly monitored on regular bases. This should be done because the presence of high microbial population in honey indicates the non-hygienic condition and lack of safety of the product been used to treat various ailments and for consumption especially when children under 12 months are involved [9,25]. The identification of *bacillus* spp such as *Paenibacillus* in honey is very important because some of these species of *Bacillus* can cause disease such as American foulbrood which is a disease of high socioeconomic importance and significant in international trade. The spores are highly resistant to heat and chemical agents and can survive in the environment for several years. Only the spores are capable of inducing the disease when found in honey. A study carried out in Argentina involving 433 honey samples reported the presence of 27% *B. cereus*, 14% yielded other *Bacillus* species. The *B. cereus* obtained from Argentinean honey were compared with isolates from honey from other countries using rep-PCR fingerprinting with primers Box, REP and ERIC, restriction fragment length polymorphism analysis of a 16S RNA gene fragment and morphological and biochemical tests. The result showed high diversity, both phenotypic and genotypic among the isolated *B. cereus* [26]. The results obtained in this study are also in accordance with studies carried out by Lurlina and his colleagues who revealed the presence of *Bacillus* species in 28 honey samples out of 70 [27].

For organisms in honey to be identified correctly different methods involving morphological examination, biochemical and molecular studies have to be used to obtain accurate results. Identification based on rRNA gene sequence fails to distinguish one species from the other if they share highly similar rRNAa genes, other genes such as recA and DNA has been employed instead of rRNA genes [25,28]. It is therefore necessary to compare other methods of identification for confirmation.

3. CONCLUSIONS

The study revealed that honey has the ability to support the growth of microorganisms even as it has antimicrobial activity. These microorganisms associated with honey cannot be identified by morphological and biochemical methods alone. Current methods for molecular identification of microorganism should be carried out alongside morphological examination and biochemical methods of identification.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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