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Microbiological and Physico-chemical Examination of Some Honey Samples from Southwest, Nigeria.

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ABSTRACT

Honey samples have been characterized in different parts of the world and have showed to possess healing potentials with health promoting capacity. The microbiological and physico-chemical characteristics of honey samples from southwest zone of Nigeria were studied. The isolated micro-organisms were bacteria, Enterobacteria (Klebsiella pneumonia, Escherichia coli, Enterobacter aerogenes); Pseudomonas Spp, (Pseudomonas pudida, pseudomonas cepacia, pseudomonas fluorescens, pseudomonas chlororaphis); Bacillus spp, Staphylococcus Spp (S. saprophyticus, S. aureus); Aeromonas hydrophilia and fungi, Aspergillus Spp and Candida Spp. The physico-chemical analysis showed that pH of honey samples ranged from 5.00-5.50, while the viscosity ranged from 48.60-89.50 at 200C. The ranged obtained for other parameters were 0.471-0.635for specific gravity; 62.10-77.80 for percentage total reducing sugar; 18.75-29.30 for percentage moisture content; while the range for percentage mineral contents for calcium and potassium were 1.11-3.18 and 1.30-3.80 respectively. Growths were obtained on almost all media used, with the microbial load of Coliform ranging from 1.2-7.2x101, while the heterotrophic count ranged from 0.8-5.4x101. The count of Salmonella Shigella Agar (SSA) ranged from 0.2-3.6x101 while the yeast-mould count ranged from 0.2-6.0x101. Hazard analysis of the products and processing of honey showed the occurrence of numbers of microbial contaminants could be due to contamination from secondary sources (dust, wind, processing equipment or materials, handlers, processing room or from the environmental sources (storage room, marketing environment and collection environment (farm). The antibiotic sensitivity testing of the isolates showed that a number of the bacteria isolates were resistant to antibiotics with the number of such resistance ranging from 3-8 antibiotics.

Keyword: Honey, microbiological, physico-chemical and antibiotic analysis.

INTRODUCTION

Honey is the natural sweet substance produced by honeybees from the nectar of blossoms or secretion of living parts of plants or excretion of plant-suckling insects living on parts of plants which honeybees collect, transform and combine with specific substances of their own, store and leave in the honey comb to ripen and mature [1]. Honey has numerous uses and functional applications worldwide such as in food systems, religious and magical ceremonies as well as in human and veterinary medicine [2,22,25]. It is a complex mixture which presents a very great variation in composition and characteristics due to its geographical and botanical origin [3,5,24], its main features depend on the floral origin or the nectar foraged by bees. Honey is composed primarily of the sugars, glucose and fructose; its third greatest component is water. It also contains numerous types of sugars, as well as acids, proteins and minerals [7,8]. It also contains a variety of phytochemicals as well as other substances like organic acids, vitamins and enzymes that may serve as sources of dietary antioxidants, and the predominant enzymes are diastase (amylase), invertase (α-glucosidase) and glucose oxidase [9,10]. The amount and type of these antioxidant compounds depends largely upon the floral source or variety of the honey [9]; darker honeys possess higher antioxidant contents than lighter honeys. However, most of the antimicrobial activity of honeys occurs due to hydrogen peroxide generation and the flavonoids in honey, particularly caffeic acid and ferulic acid, as the most likely contributors [5,21,22]. Bacteria and fungi have been reported to grow and ferment or spoil unprocessed honey [5], although proper processing and handling can control spoilage. Therefore, the microbiological quality of honey may serve as an indicator of the hygienic conditions under which the product is processed, handled and stored. The shelf-life of honey or tendency to crystallize is directly related to its glucose and water contents, origin, age, and storage conditions [4,23,24]. Since honey is a 'wonderful rich golden liquid' available everywhere for consumption, medicinal, therapeutic, traditional and industrial uses [36], it is therefore of importance to evaluate the sterility of both raw and processed honey in the environment and to ensure the safety of its application. Thus, this study seeks to investigate the microbial analysis of honey samples collected from different states in the South West of Nigeria in order to determine the physico-cochemical characteristics of the collected honey samples, microbilological analysis of honey samples and the public health implication of the isolates through sensitivity testing.

MATERIALS AND METHODS

Study Area and Collection of Samples

Honey samples were collected from the following states; Ondo, Ekiti, Oyo, Osun, Ogun and Lagos. A total of 30 honey samples were collected, five of the honey samples were processed honey bought from retailers while 25 samples were raw honey locally collected by farmers. Some were collected directly from the honey hives into a sterilized bottle, while some were already bottled and sold by farmers and venders. In some cases, sterile bottles were used to collect honey samples from farmers and were labeled 1-30.

Sampling area

The honey samples were collected from farms, markets, road sides, stores and supermarkets. The processed honey samples were bought from stores and supermarkets with good sanitation level while the road sides and market honey was bottled and displaced in open where patronage of buyers is ensured. The road sides and market are characterized with low level of sanitation, with lots of human activities.

Physico-Chemical Analysis of Honey Samples

The physico-chemical analysis of 12 honey samples were determined and the parameters measured include: pH, specific gravity, viscosity, % moisture content, %total reducing sugar, % ash content and % crude protein. The pH was measured using a pH meter [37]; density was calculated using a mass to volume ratio, while viscosity was calculated using the ratio of drop of a sphere of known weight and dimension through the honey sample. The moisture content was obtained by drying 2 grams of each honey sample in a hot air oven at 100% for 24 hours until a constant weight was attained, and the ash content was determined by incinerating dried samples in a muffle furnance at 550 ^{0}C for 4 hours and cooled at room temperature and weighed. The atomic absorption and spectrophotometer method as described by Pauwels et al.,[38] and was used to analyse the content of calcium, magnesium, iron and zinc at 240nm, 285.2nm, 283.3nm and 285.2nm respectively. The content of potassium was analyzed using flame photometer while phosphorus content was determined at 470nm using spectromic 21D. The crude protein content and colour were also analyzed.

Microbiological Analysis of Honey Samples

Honey samples were subjected to microbiological analysis according to conventional method. The standard plate count method was used for culturing and isolating the different microorganisms. 1ml from each honey samples was taken and serially diluted in sterilized water of appropriate dilution of 10, 100, 100, 10000th folds and 0.5ml of the 10^{-2} diluents was used for enumeration and isolation of microorganisms are: MacConkey Agar (MA), Salmonella-Shigella Agar (SSA), Nutrient Agar (NA) and Potato Dextrose Agar (PDA). All plates that were incubated at 37^{0}C for 24-48 hours, except the PDA plates that were incubated at room temperature (30±2^{0}C). The isolation and culturing of bacteria using MA, SSA and NA were carried out with the aim of using MA isolate coliform bacteria, SSA for Salmonella and Shingella species and NA for heterotrophic or aerobic bacteria. At the end of incubation, the number of distinct colonies were counted and used to calculate the microbial load in each case. Thereafter, colonies were purified to obtain pure cultures and then stored on agar slants at 4^{0}C. After successive transfers, the resulting pure isolates were Gram stained and identified based on the colour, sizeand shape [39].

Identification of Isolated Microorganisms

Biological and morphological characterization of the isolates were carried. Different tests such as catalase reaction, oxidase test, casein hydrolysis, gelatin hydrolysis, methyl red test, nitrate reduction, coagulate test, urease test, starch hydrolysis, citrate test, motility and indole test were used for identification of organisms. Also, fermentation of different sugars such as glucose, fructose, maltose, lactose, sucrose, galactose, xylitol, arabinose, raffinose and mannose were used for the identification of the isolates.

Antibiogram of Bacterial Isolates

Sixteen strains of bacterial isolates were tested for different sensitivity to antibiotics by means of M2-A6 disc diffusion method (40), using Nutrient agar. Antibiotic discs (Fondoz laboratory) containing:Augmentin (Aug), 30µg; Amoxycillin (Amx), 25µg; Ofloxacin (Ofl), 5µg; Tetracycline (Tet), 30µg; Ceftriazone (Cro), 30µg; Nitrofurantoin (Nit), 200µg; Gentamycin (Gen), 10µg and Pefloxacin (Pfx), 5µg were used for Gram negative isolates. Amoxicillin (Amx), 25µg; Gentamicin (Gen), 10µg; Cotrinoxazole (Cot), 25µg; Erythromicin (Ery), 5µg; Stretomycin (Str), 10µg; Ciprofloxacin (Cpx), 10µg; Ceftriazone (Cro), 30µg; Chloramphenicol (Chl), 30µg; Pefloxacin (Pef), 10µg and Ofloxacin (Ofl), 5µg were used for Gram positive isolates. The plates were incubated at 37^{0}C for 12-24 hours. After incubating, zones of inhibition were examined and interpreted accordingly (41) considering the appropriate breakpoints.

Identification of Hazards and Critical Points in the Production and Processing of Honey

Identification of hazards and critical control point during the production and distribution of honey was carried out through visitation to two honey farms (Pa Osakuade honey farm, Ilaramokin and Sunshine honey farm, Ondo road akure) and one honey processing factory (Sunshine honey factory, Alagbaka, akure). Production and packaging of the honey was observed locally on the farm and industrially in the factory, while the sellers and selling areas were also monitored regarding the display of honeys for sales. Several other parameters were monitored, amongst which are the hygienic condition of the farm and production environment as well as containers or bottles used for packaging and storage of honey. All these were done to identify the sources of contamination in the production and marketing chain of honeys.

RESULTS

Twelve honey samples were selected for physico-chemical analysis as presented in Table 1. The pH of the honey samples varied from 5.00 – 5.50, while the specific gravity ranged from 0.471 – 0.635. The viscosity ranged from 48.50 – 89.50; total reducing sugar ranged from 62.10 - 77.80; percentage moisture content ranged from 18.75 – 29.20%. The percentage mineral content of calcium and potassium ranged from 1.11 – 3.18 and 1.30 – 3.80 respectively.

The microbiological analysis of the honey samples is presented in Table 2 showing a microbial contamination with index of 101. The coliform counts ranged from 1.2 – 7.2x101, while the heterotrophic count ranged from 0.8 – 5.4x101. The count on SSA ranged from 0.2 – 3.6x101, while the yeast – mould count

ranged from 0.2 – 6.0x101. However, growths were obtained on SSA in four samples. A total of 23 bacterial isolates were encountered during the analysis. Details of the bacterial isolates and their sources are presented in table 4.3. The bacterial isolates are Enterobacteriaceae (Klebsiella pneumonia, Escherichia coli, Enterobacter aerogenes); Pseudomonas spp. (Pseudomonas pudida, Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas choloraphis); Bacilus spp, Shigella spp, Staphylococcus spp (Staphylococcus saprophytisus, Staphylococcus aureus); Aeromonas hydrophila. Fungal isolates included Aspergillus spp. and Candida spp. The antibiogram of the isolates is presented in Table 4 all isolates showed drug-resistance, with the numbers of antibiotics ranging from 2 -8.

Table 1.Physio-chemical attributes of some of the honey samples

Physico-chemical attributes	Honey samples											
	1	2	3	4	5	6	7	8	9	10	11	12
pH	5.40	5.20	5.40	5.20	5.50	5.30	5.40	5.30	5.30	5.40	5.40	5.00
Specific gravity	0.471	0.521	0.500	0.498	0.610	0.590	0.550	0.540	0.510	0.520	0.570	0.635
Viscosity @ 20^0C (Ns/m^2)	64.30	65.10	55.10	50.10	76.50	80.30	50.10	56.70	48.60	50.80	75.20	89.50
Total reducing sugar	68.50	67.10	71.40	73.10	77.10	77.80	75.90	76.30	76.40	74.60	75.10	62.10
% ASH	0.12	0.10	0.07	0.07	0.05	0.07	0.10	0.10	0.08	0.08	0.11	0.03
% moisture content	24.70	24.80	27.10	28.80	21.20	20.40	28.70	26.70	29.30	28.70	21.40	18.70
% crude protein	0.42	0.38	0.35	0.33	0.40	0.40	0.37	0.34	0.30	0.34	0.38	0.45
% mineral content												
Potassium (k)	2.20	2.14	3.80	2.80	2.28	1.30	2.20	2.50	2.14	2.10	2.86	3.20
Calcium (Ca)	2.13	2.10	2.83	2.14	1.09	1.11	2.06	2.36	1.18	2.10	2.60	3.18
Phosphorus (P)	0.07	0.08	0.09	0.10	0.13	0.08	0.08	0.09	0.09	0.12	0.13	0.14
Magnesium (Mg)	0.08	0.06	0.07	0.08	0.07	0.012	0.04	0.04	0.04	0.08	0.017	0.153
Iron (Fe)	0.04	0.04	0.12	0.10	0.10	0.12	0.11	0.11	0.13	0.05	0.06	0.26
Zinc (Zn)	0.12	0.03	0.08	0.14	0.99	0.97	0.05	0.05	0.05	0.018	0.029	0.13

Honey samples with Physico-chemical attributes and percentage mineral content.

Table 2. The microbial loads of the honey samples

Samples/microbial load (cfc/ml x 10^1)	MA	SSA	NA	PDA	Total microbial counts
1	2.4	0.2	4.6	0.8	8.0
2	3.2	0.6	3.4	0.2	7.4
3	7.2	1.2	2.6	2.0	12.0
4	1.2	0.2	4.0	1.8	7.2
5	5.6	0.6	2.0	1.4	9.6
6	3.8	1.8	5.4	2.8	19.0
7	3.2	0.6	3.2	3.0	10.0
8	1.6	2.8	1.2	2.0	7.6
9	2.0	0.8	2.6	2.2	7.6
10	3.6	-	1.8	1.8	7.2
11	4.8	1.0	2.0	6.0	13.8
12	5.6	-	3.0	4.8	13.4
13	4.4	2.4	2.2	1.8	10.8
14	2.4	0.6	1.8	0.6	5.4
15	2.8	1.2	1.4	3.0	8.4
16	4.6	2.0	2.6	1.6	10.8
17	6.0	1.8	2.4	0.6	10.8
18	5.6	-	1.4	2.4	9.4
19	1.8	3.2	2.0	2.4	9.4
20	2.6	2.8	1.6	1.2	8.2
21	1.2	1.0	0.8	0.6	3.6
22	2.6	1.4	2.0	3.0	9.0
23	2.0	2.6	1.8	3.2	9.6
24	2.2	-	2.2	4.8	9.2
25	2.4	3.6	2.8	5.0	13.8
26	3.0	2.4	2.6	4.4	12.4
27	1.8	1.6	4.0	2.0	9.4
28	1.4	2.0	3.8	1.6	8.8

29	1.2	2.4	1.6	1.0	6.2
30	2.8	1.8	1.0	0.8	6.4

Total microbial load, counts and samples of honey.

Table 3. The Microbial isolates obtained from the honey samples.

S/N	Isolates	Gram reaction	Morphology	Sources
1	*Klebsiella pneumonia*	-	Rod	7,11,22
2	*Enterobacter aerogenes*	-	Rod	6,12,2,27
3	*Aeromonas Hydrophila*	-	Rod	2,20,14,17
4	*Pseudomonas putida*	-	Rod	4,3,11,28
5	*Pseudomonas fluorescens*	-	Rod	12,4,2,7,6,20
6	*pseudomonas cepacia*	-	rod	10,11,13
7	*Pseudomonas chlororaphis*	-	Rod	5,1,10,3
8	*Staphylococcus aureus*	+	Coccus	1,4,11
9	*Staphylococcus aureus*	+	Coccus	7,11,15
10	*Shigella spp*	-	Rod	6,9,20
11	*Bacillus spp*	+	Rod	1,4,7,11,17,12
12	*Aspergillus niger*		Filamentous	1,3,9,11,17
13	*Candida spp*		Oval	11,4,7,5,8,13

Gram reaction of honey sample isolates with their sources and morphology.

Table 4. The antibiogram of bacterial isolates obtained from the honey samples

Numbers of antibiotics	Resistance patterns	Isolates
2	Cro, Gen	*Shigella sp*
3	Cro, Aug, Tet Amx, Cro, Ery	*K. pneumonia, P. Cepacia* *Staphylococcus spp*
4	Aug, Cro, Cot, Tet Amx, Cro, Aug, Tet Amx, Cot, Gen, Tet Amx, Gen, Nit, Tet Amx, Cro,Cot, Aug	*p. cepacia* *E. coli* *Enterobacter aerogenes* *Pseudomonas fluorescens* *Pseudomonas fluorescens*
5	Amx, Cot, Cro, Gen, Ery Amx, Nit, Gen, Cot, Aug Amx, Nit, Gen, Cot, Aug	*Staphylococcus spp* *Aeromonas hydrophila* *Pseudomonas fluorescens*
6	Amx, Cro, Ery, Aug, Cot, Chl Amx, Chl, Cro, Gen, Cot, Ery Amx, Cro, Nit, Gen, Aug, Tet Amx, Cro, Nit, Cot, Aug, Tet	*Staphylococcus spp* *Bacillus spp.* *K. pneumonia* *P.chlororaphis*
7	Amx, Cro, Gen, Cot, Nit, Aug, Tet Amx, Cro, Gen, Cot, Nit, Aug, Tet Amx, Cro, Gen, Cot, Nit, Aug, Tet AMx, Str, Chl, Cro, Gen, Cot, Ery	*P. cepacia, P.pytida, P. Fluorescens* *E. coli, E. Aerogenes* *Aeromonas Hydrophila* *Bacillus spp., S. saprophyticus*
8	Amx, Cro, Cot, gen, Ofl, Tet, Nit, Cpx	*Enterobacter aerogenes*

Abbreviations: Amx, Amoxycyllin; Ofl, Ofloxacin; Str, Streptomycin; Chl, Chloraphenicol; Cro, Ceftriaxone; Gen, Gentamycin; Pfx, Pefloxacin; Cot, Co-trimoxazole; Cpx, Ciprofloxacin; Ery, Erythromycin; Aug, Augumentin; Nit, Nitrofurantoin; Tel, tetracycline.

However, the production and processing of honey involved different steps through which microbial contamination can find their ways into honey (Table 4). Some of these sources include raw materials (nectar and pollens), frames, processing equipments, handling rooms and processors, however, these hazards can be abated by applying a numbers of control measures such as regular cleaning and disinfection of wooden frame, improved hygiene during collection, extraction, processing and food manufacturing.

Table 4.5: The processing steps, sources of hazards and control measures in the production of honey

Processing steps	Sources of hazard	Hazard	Control measures
Deposition of nectar/ pollen in hives of bees	Raw materials (nectar, pollens), bees, hive	Pathogenic microorganisms and dirt	-
Placements of frames in	Frame	Pathogenic	Clean the wooden frames before

hive		microorganisms	use
Removal of frame from hive	Soil/air, hand, container	Pathogenic microorganism	Proper hygiene and use of clean container
Extraction (local extraction)	Knife, container, hand, exposure of comb to sun for easy flow	Pathogenic microorganisms and dirt	Clean knife and heat first before use, Extract honey inside a clean container than has cover with muslin cloth and then place under sun. Proper hygiene.
Industrial extraction	Extractor, honey handling room, water	Pathogenic microorganisms	Quality assurance and inspection. Regular cleaning of extractor and proper hygiene. Use of potable water.
Pumping and filtration	Pipe/pumping machine, Sieve/Mesh and container/vessel	Pathogenic organism	Use of clean and disinfected sieve/mesh, vessel/ container. Good quality assurance and inspection. Regular cleaning and disinfection of flow pipes.
Treatments (only in industrial process) -Heating	Product deterioration occur at heating above 75^0C	*Increase the level of Hydroxymethyl-furfural. *Reduces enzymes *Affects sensory quality *Reduces the freshness of the honey *cannot effectively destroy heat resistance organisms	*(locally) exposure to sunlight melt honey for easy flowing through extractor and filter. *use of honey warming kit (it has a thermometer and fan) *Use of an insulator cabinet *for destruction of microorganisms, - heating of honey at 70 - 71^0C for 2mins followed by rapid cooling to 54^0C - heating of honey at 60^0C for 30mins *non thermal alternatives are preferable; e.g ultrasonication or microwave radiation can be Used of destruction of microorganism
Mixing and bottling	Mixing stick, vessel, bottles or container, handling room and hand	Pathogenic microorganisms	Proper hygiene, Quality assurance and inspection, Bottle/container sterilization (if glass) but wash properly in potable water if not.

Steps involve in honey processing, hazard and control measures.

DISCUSSION

In the present study the physico-chemical indices of honey samples showed that there were wide variation in the values of viscosity, moisture content, specific gravity, water content, sugar content, crude protein, mineral content and ash content. The pH values of the honey sample fall within the standard reference range given by the National Honey Board (www.honey.com) and this indicate that the sample are mildly acidic. Honey samples' viscosities were between the ranges of 48.60 – 89.50Ns/$m^{2.}$ These parameters are dependent upon water content, temperature and floral sources [23,24]. The southwest region of Nigeria is at an annual temperature range of 18 – 35^0C, annual evaporation rate range of 0.20 – 1.00mm. The water content of honey samples from this region range from 18.75- 29.30%. The exact floral sources of southwest honey are not fixed due to a mixture of vegetation in the region. The viscosity of the honey samples fall within the range of honeys from western highland of Cameroun whose annual temperature falls within 16 – 27^0C [26].

Minerals are usually present in very small quantities in honeys; potassium being the most abundant and dark honeys being the richest in mineral content [26]. Although the southwest Nigeria honey samples also contained many minerals in trace quantities, calcium and potassium were both more abundant. The colour of the honey samples used in this study varied from light amber to dark amber.

The result of the microbial analysis of the honey samples showed that, almost all the honey samples are contaminate with bacteria and fungi. In most cases, the microbial load of the honey samples varied with the 'raw honey' samples having

higher microbial load compared to the 'processed honeys' that have undergone industrial processes. The bacterial isolates were mainly Bacillus spp., Shigella spp., Klebsiella pneumonia, Pseudomonas spp *(Pseudomonas pudida, Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas choloraphis), Staphylococcus spp (Staphylococcus saprophytisus, Staphylococcus aureus); Aeromonas hydrophila*. While the Fungal isolates are *Aspergillus spp*. and *Candida spp*. These correlate with several result got from microbial analysis of honeys in Nigeria [27]; Cameroun [26], Saudi Arabia [28]. They demonstrated the presence of Bacillus spp., *Enterobacteriaceae, Pseudomonas spp., Staphylococcus spp., Aspergillus spp.* and *Candida spp*. in honey.

However, the result of the antibiogram of bacterial isolates showed that a variety of resistance pattern were observed in this study and among all bacteria tested. Resistance was higher to Amoxicillin, Erythromycin, Tetracycline, Cefriaxone, Co-trimoxazole, Gentamycin, Nitrofurantoin, Chloramphenicol, Augumentin and Streptomycin while little or no resistance was found to Ofloxaxin, Ciprofloxacin and Pefloxacin. The high level of resistance seen in Amoxicillin and tetracycline correspond to what has been found in many studies in a number of countries [29,32].

Total resistance of Amoxycillin and Augumentin in Aeromonas hydrophila in this study is also in accordance with what has been reported commonly in Aeromonas spp., which is due to the production of β-lactamses by this organism[33]. The relatively high resistance of bacteria pathogen to antimicrobial agents in this study agrees with literature reports by Barat *et al*., [42]; [29,30].

Resistance to ofloxacin and ciprofloxacin is of low frequency in this study, as there is little or no resistance to this antibacterial agent. Fluoroquinolone resistance has been reported in environmental isolates at a relatively low frequency [34,35]. Many of the isolates in this study showed multiple antibiotics resistance. All isolates are resistant to more than one class of antibiotics indicating that there is emergence of multi-resistant strains.

CONCLUSION

Based on the microbial population observed in the honey samples, it could be stated that honey samples collected from local markets of southwest Nigeria are contaminated with fungi and bacteria organisms indicating a major secondary contamination and inadequate hygiene condition during harvesting, handling, processing and/or storage. Therefore, honey may serve as reservoir for microorganisms and play a role (to some extent) as a causative agent of some foodborne illnesses especially in children and immune-compromised individual. The safety of the honey samples for human commercial consumption is doubtful.

The occurrence of microorganisms with high resistance to these antimicrobial agents might pose therapeutic problem as well as health to honey consumer due to the spread of resistant bacteria from the environment to human.

RECOMMENDATION

Honey pastes production should be controlled and inspected by both health and standardization authorities. Farmers, managers and owner of factory from processing honey pastes should be encouraged to apply good manufacturing practices through application of total quality programs including hazard analysis and critical control points system. Consumers should be aware when consuming honey pastes regarding their sources and contents. It is advisable to consumers to purchase processed honey or freshly comb honey due to the extremely low microbiological quality. It may also be important to determine the molecular basis of the obse3rved resistance in the organisms and the possible transfer of such organisms to other bacteria species.

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