International Journal of Probiotics and Prebiotics Vol. 11, No. 2, pp. 85-98, 2016
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THERAPEUTIC EFFECT OF LACTIC ACID BACTERIA ISOLATED FROM FRESHLY- HARVESTED HONEY AND ITS COMB AGAINST FOOD AND WOUND BORNE –PATHOGENS

[1]S.M. Adeyemo, [2]F.T. Afolabi, and [3]E.D. Ogunlusi

[1,3]Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria; and [2]Department of Microbiology, University of Ibadan, Ibadan, Nigeria.

[Received February 17, 2016; Accepted June 2, 2016]

[Communicated by Prof. Francesco Marotta]

ABSTRACT: ***Honey has been extensively studied in the treatment of wounds but its efficacy in clinical practice is not fully understood. Lactic acid bacteria (LAB) have been used as bio-preservative in fermented foods. This work was carried out to evaluate the antagonistic effect of LAB present in fresh honey and its comb against some pathogens. LAB were isolated from five replicate samples of freshly harvested honey and its comb and identified as Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus plantarum and Lactobacillus lactis. Lactic acid, Diacetyl and Hydrogen peroxide (g/l) production were determined for 5 days. L. plantarum and L. brevis exhibited the highest and the lowest antimicrobials production, respectively. Agar well diffusion method was used to monitor the antagonistic activities against some pathogens. L. plantarum and L. brevis gave the highest zones of inhibition ranging from15-23mm on P. aeruginosa and S. aureus from food samples. They compete favourably with standard antibiotics set up as controls. Lactic acid bacteria demonstrated a significant antimicrobial activity; this shows its potential as probiotics in food preservation and its therapeutic effect in wound treatment. This also justifies the efforts directed by individuals in using honey to treat diabetic wounds.***

KEYWORDS: Antagonistic activities, Antimicrobial compounds, Food borne pathogens, Honey, Lactic acid bacteria.

Corresponding Author: S.M. Adeyemo, Food and Industrial Microbiology Unit, Department of Microbiology, Obafemi Awolowo University, Ile Ife, Osun State, Nigeria. Telephone: +234 (0) 706 970 0215; E-mail: adeyemostella@gmail.com.

INTRODUCTION

From ancient times, honey was not only used as a natural sweetener but also as a healing agent. Many health-promoting and curative properties attributed to it are the basis for some traditional folk medicine treatments throughout the world today. Of the consumers who use honey, ninety-three percent consider honey a healthful product, recognizing it as a pure, natural product. Fifteen percent think of it as a good home remedy (National honey board, 1997). Moreover, a number of characteristics of honey contribute to its antimicrobial activity. The enzymatic glucose oxidation reaction and some of its physical properties are considered to be the major factors. Other factors include high osmotic pressure/low water activity (Aw), low pH/acidic environment, low protein content, high carbon to nitrogen ratio, low redox potential due to the high content of reducing sugars, a viscosity that limits dissolved oxygen and other chemical agents or photochemicals (Snowden and Cliver, 1996). Some Honey researchers,including Molan (1999) has written an extensive review of their research on the antimicrobial factors in honey. He summarized the key aspects of his research in his different reports (Molan, 1999, 2002 and 2009).

According to Molan (2009) and other researchers, honey is a supersaturated sugar solution with a low water activity (Aw), which means that there is little water available to support the growth of bacteria and yeast. They opined that the natural acidity of honey would inhibit many pathogens. The minimum pH value for some species that commonly infect wounds ranges from 4.0-4.5. Dilution of honey, especially with body fluids, will raise the pH and lessen the antibacterial effectiveness that results from its acidity (Snowden and Cliver, 1996; NHB 1997; Malike *et al.*, 2004; Molan, 2009).

In addition, several authors explained in their work that Glucose oxidase is an enzyme secreted by the bees to form

honey from nectar. This converts glucose in the presence of water and oxygen to gluconic acid and hydrogen peroxide. The resulting acidity and hydrogen peroxide preserve and sterilize the honey during the ripening process. Full-strength honey has negligible amounts of hydrogen peroxide and active glucose oxidase. Transition ions and ascorbic acids rapidly decompose hydrogen peroxide to oxygen and water while the low pH inactivates the enzyme. However, dilution of honey results in a 2,500-50,000 increase in enzyme activity and a "slow-release" of antiseptic that does not damage tissues (Snowden and Cliver, 1996; NHR 1997; Malike *et al*., 2004; Molan, 2009).

Lactic acid bacteria (LAB) on the other hand display numerous antimicrobial activities in the fermented foods. This is mainly due to the production of bacteriocins, organic acids,ethanol, Hydrogen peroxide, Diacetyl and reuterin. Lactic acid bacteria play an important role in food industry by increasing nutritional values of food and food safety (Zhennai, 2000; Adeyemo and Onilude, 2013 and 2014). The antimicrobials produced by LAB have been used widely asbio-preservatives and shelf life extenderand has found application in many industries and various commercial purposes. *Lactobacillus spp* is the main genus of LAB and itplays an important role in balancing microflora inthe gut ecosystem of human and animals. LAB has also been isolated from freshly harvested honey and its comb (Lee, 2002; Brashears *et al*., 2005; Canibe *et al*., 2007).

Recently, it was reported that, bacterial metabolites also display a wide inhibitory activity against various pathogens such as *Escherichia coli, Salmonella typhimurium and Pseudomonas aeruginosa* (Savadogo *et al*., 2006; Gaggìa *et al*., 2010; Thanh *et al*., 2010).Nowadays people are faced with alot of health problems caused by their life style.Human cardiovascular disease is the most important problem in many countriesdue to hyper cholesterol levels (Kim *et al*., 2008). LAB such as probiotics has a long history of application and consumption by human as safe (Holzapfel *et al*., 2001).

*P.aeruginosa*and *S. aureus* are ubiquitous bacterial strain which may occur in soil, animal or human skin or other parts such asfeces, water food etc. They are associated with different diseases and infections in man and animals especially patients with low resistance whose immunesystem has been compromised or suppressed. They also invade burns, wounds and cause urinary tract infections(Nudda *etal*., 2006). A variety of selective and differential media has been proposed, particularly for the isolation and identification of these organisms especially from clinical, environmental, and food samples and their resistance to major antibiotics and their patterns. Some problems may arise especially when quantitative recovery is required or in cases where large numbers of competing flora are present (Holzapfel *et al*., 2001; Nudda*et al*., 2006).

The aim of this work is to evaluate the antagonistic effect of lactic acid bacteria present in fresh honey and its comb against Gram positive and negative bacteria isolated from food and wound samples. It also involves the use of the probiotics lactic acid bacteria as a source of novel antibiotics and their therapeutic effects against these antibiotics resistant organisms.

MATERIALS AND METHODS

Sample Collection

Five replicate samples of freshly harvested honey and its comb were purchased at different locations in Osun State, Nigeria. They were transported to the Laboratory in sterile containers for further analysis.

Culture Media and Isolation of Organisms

MRS (de ManRogosa and Sharpe) broth and agar medium, Nutrient agar, and Peptone water were prepared and homogenized according to the manufacturer's instructions. Sterilization of medium was carried out at 121°C for 15mins, and cooled to a temperature of 45°C before pouring. The method of (Harrigan and McCance, 1998) was used for the isolation. The plates were left to solidify, inoculated in triplicates, and inverted. MRS plates were incubated at30°C for 48hours under anaerobic conditions. After the incubation period, representative colonies of various isolates were selected at random and sub-cultured repeatedly to obtain a pure culture.

Culture Preservation

Pure cultures of LAB isolates were subcultured into MRS agar slantsand incubated at 30°C until growth becomes visible. It was then overlaid with glycerol and kept at 4°C in the refrigerator for further use.

Characterization of the Test Isolates

Characterization of isolates was carried out by employing macroscopic, microscopic and standardbiochemical tests (Olutiola *et al*., 1991).

Macroscopic Examination

The isolates were examined for growth type, shape, elevation, size, margin pigmentation and consistency.

Inoculum Preparation

The inoculum was prepared by inoculating a colony picked from each of the LAB plates into a sterile 9ml MRS broth using a sterile inoculating loop. The inoculated test tubes were incubated for 5 days and the antimicrobial compounds produced were determined ondays 0, 3, and 5 respectively. The pH of the growth culture was also recorded daily.

Preparation of Cell Free Culture of LAB

The culture broth for antimicrobial activities was prepared separately; this was centrifuged at 10000 rev/min for 15 minutes. The cell free culture supernatant was labeled as the supernatant. This was used for the antimicrobial susceptibility test.

Determination of Lactic Acid productionby the Isolates.

Estimation of lactic acid produced was determined by

titration of 25ml of broth cultures of the test organisms (24hr old) with 0.1N NaOH. 3 drops of phenolphthalein were added as indicator. NaOH was then added slowly to the sample until a pink colour appeared. Each ml of 0.1N NaOH is equivalent to 90.08mg of lactic acid as stated in A.O.A.C (2000).

$$\text{Titratable acidity of lactic acid} = \frac{\text{mlNaOH} \times \text{N NaOH} \times \text{M.E} \times 100}{\text{Volume of sample used}}$$

ml NaOH = Volume of NaOH used
N NaOH – Normality of NaOH
M.E = Equivalenat factor = 90.08mg
A.O.A.C (2000)

Determination of Diacetyl production by the Isolates

Diacetyl produced by the isolates was estimated by measuring 25ml of the broth cultures of the test isolates (24hrs) into conical flasks and 7.5ml hydroxylamine solution was used for residual titration. The flasks were titrated with 0.1N HCl to a green-yellow end-point using bromophenol blue as indicator. The equivalent factor of HCl to diacetyl is 21.52mg.

$$Ak = (b - s)(100 - e)/w$$

Ak = Percentage of diacetyl
b = Number of ml of 0.1N HCl consumed in titration of the sample
e = Equivalence factor = 21.52mg
w = Volume of sample
s = Number of ml of 0.1N HCl condensed in titration of residue sample
A.O.A.C(2000).

Determination of Hydrogen Peroxide production by the Isolates

About 20ml of diluted H_2SO_4 were added to 25ml of the broth cultures of the test organisms (24hrs). Titration was carried out with 0.1N $KMnO_4$. Each ml is equivalent to 1.70mg of H_2O_2 and declourization of the sample was regarded as the end point.

$$H_2O_2 \text{ Concentration} = \frac{\text{ml}KMnO_4 \times NKMnO_4 \times \text{M.E} \times 100}{\text{Ml } H_2SO_4 \times \text{Volume of sample used}}$$

Ml $KMNO_4$ = Volume of acid used
$NKMnO_4$ = Normality of $KMnO_4$
Ml H_2SO_4 = Volume of H_2SO_4 added
M.E = Equivalent factor = 1.70mg(A.O.A.C, 2000).
***The tests were carried out in replicates.

Standardization of Test Organisms

The test microorganisms were standardized by using 0.5 McFarlandstandards. 0.5 McFarland gives approximate cell density of 3.0 x 10^3Cfu/ml andoptical density of 0.500 at wavelength of 600 nm. The microbial suspensions were prepared in their respective sterile nutrient broth and were compared with that of the standard (Andrews, 2001).

Antimicrobial Susceptibility Testing

Samples with high yields of antimicrobial compounds were selected for the antimicrobial susceptibility test. 3ml from each of the samples was transferred into 10ml of a fresh sterile MRS broth and incubated for 72 hours. The samples were centrifuged at 10,000rpm and decanted to remove the cells.1 ml of standardized inoculums was introduced into the plates and the plates were replicated (*P. aeruginosa* and *S.aureus*). Sterile nutrient agar of about 20mls was poured on plates and allowed to solidify. Holes were bored in the plates at 4 different angles using a 6 mm diameter cork borer. 1ml of the LAB cultures were introduced into the holes. Controls were set up with uninoculated MRS broth, standard Antibiotics and honey. Plates were then incubated for 18hours and the zones of inhibition recorded.

Statistical Analysis

Data obtained were subjected to ANOVA and Duncan Multiple Range of Variables.

RESULT AND DISCUSSION

Samples of freshly harvested honey and its comb were used in this study. A total of 12 LAB isolates were obtained and identified as *L. casei*, *L. brevis*, *L. acidophilus* and *L. plantarum*. The percentages of occurrence of these isolates obtained are*L. brevis* (33.3%), *L. plantarum* (33.3%), *L. casei* (16.7%) and *L. acidophilus* (16.7%).

The antimicrobial production of Lactic acid, diacetyl and hydrogen perioxide of *L.casei, L. plantarum* and *L. brevis* at day 0, 3 and 5 is recorded in Table 1.It was observed that the

TABLE 1. The antimicrobial production (g/l) of Lactic acid bacteria.*Antimicrobial values of LAB expressed as mean of three determinations ±standard deviation. **Means with the same superscript are not significantly different. **Key**: L.A, D.A and H.P represent Lactic acid, Diacetyl and Hydrogen peroxide.

DAYS	0 (H.P)g/l	3 (H.P)g/l	5(H.P)g/l	0 (L.A)g/l	3 (L.A)g/l	5 (L.A)g/l	0 (D.A)g/l	3 (D.A)g/l	5 (D.A)g/l
L.casei	**0.02^b±0.010	0.03^b±0.010	0.04^b±0.006	1.10^b±0.100	1.30^a±0.100	1.80^c±0.100	0.13^c±0.010	0.14^b±0.006	0.16^b±0.010
L.plantarum	0.03^c±0.006	0.04^c±0.006	0.05^c±0.006	1.3^c±0.100	1.8^c±0.100	1.7^b±0.150	0.13^c±0.010	0.16^c±0.006	0.17^c±0.015
L. brevis	0.01^a±0.001	0.02^a±0.010	0.03^a±0.010	1.1^b±0.100	1.5^b±0.153	1.3^c±0.058	0.11^b±0.015	0.13^a±0.006	0.17^c±0.010

production of antimicrobials increased from day 0-5 and there was a decrease after day 5.

The Antimicrobial susceptibility pattern of the LAB isolates against wound and food- borne pathogen (*P. aeruginosa*) is shown in Table 2.It was observed that *L. plantarum* gave the highest zone of inhibition (20mm), this was followed by *L.brevis* (12mm) and least was observed in *L.casei* (8mm).The Antimicrobial susceptibility pattern of the LAB isolates against food- borne pathogen*(P. aeruginosa*), *L. brevis* gave the highest zone of inhibition of (19mm), followed by *L. plantarum* (16mm) while *L. casei* (12mm) gave the least.

TABLE 2.Antimicrobial susceptibility pattern of the LAB isolates against wound- borne pathogen and food- borne pathogen (*P. aeruginosa*).*Data obtained are antimicrobial susceptibility patterns values of LAB expressed as mean of three determinations ±standard deviation. **Means with the same superscript are not significantly different.

LAB Isolates	*P. aeruginosa* (wound borne pathogen) (mm)	*P. aeruginosa*(food borne pathogen) (mm)
L. casei	**08^{a}± 1.000	12^{a} ±1.000
L. plantarum	20^{c} ±1.000	16^{b}±1.000
L.brevis	12^{b} ±1.000	19^{c} ±1.000

TABLE 3.The Antimicrobial susceptibility pattern of LAB against food- borne pathogen (*S.aureus*).*Data obtained are antimicrobial susceptibility patterns values of LAB expressed as mean of three determinations ±standard deviation. **Means with the same superscript are not significantly different.

LAB Isolates	*S. aureus* (food borne pathogen) (mm)
L. casei	**17^{b}± 1.000
L. plantarum	18^{a}± 1.000
L.brevis	12^{c} ± 1.000

The Antimicrobial susceptibility pattern of LAB against food-borne pathogen (*S.aureus*) is shown in Table 3. *L. plantarum* gave the highest zone of inhibition (18mm); this was followed by *L. casei* (17mm) and *L. brevis,* the least (12mm).

Honey is well known for its health benefits and it has been used as traditional medicine for many years (Dobrowolski*et al.*, 1991; Bankova, 2005). Honey contains ceramic acid, antioxidant agent and some flavonoids, which have been approved for antibacterial applications (Rahman *et al.*, 2010). Malika*et al.* (2004) suggested that the activity of honey varies depending on its origin, type of flowers, the region, the nature of bees, the breeding techniques and the time of harvest to its use. Zumla and Lulat (1998) reported that fresh honey is a very good inhibitor to *Escherichia coli*, *Salmonella*and *Shigella*.

During twentieth century, it was reported that fresh honey as having good antimicrobial properties along with therapeutic potential in wound healing. Honey has been studied extensively and found most effective in nearly all types of wound healing, an abrasion, abscess, amputation, burns, fistula, etc. are found to be responsive to honey therapy. Application of honey as wound dressing leads to rapid healing by stimulation of healing process, clearance of infection and cleansing action of wounds (Malika *et al.*, 2004).

In this study, a total of 12 LAB were obtained from freshly harvested honey and its comb which produced effectively various types of LABisolates. There was a challenge initially in isolating the organisms because most of the honey samples that were obtained were not fresh. LAB can be isolated from honey when they are freshly harvested. This agrees with the report of Malika *et al.* (2004). and Rahman(2010) that claimed that the activity of honey depends on origin, breeding techniques and time of harvest.

All the Lactic acid Bacteria fermented glucose, mannitol and fructose and this agreed with the work of (Holt *et al.*, 2000) who reported the ability of members of the LAB to ferment glucose and other sugar such as lactose, sucrose and maltose. All isolates grew at 20^{o}C and none grew at 70^{o}C. Some isolates thrive well at pH 9.4 which was identified as *L. casei*, *L.brevis*, *L. plantarum* and *L.brevis* respectively and after which they were chosen to check for their further antimicrobial production.

In addition, all the LAB isolates in this work produced lactic acid, diacetyl and hydrogen peroxide at varying degrees. This observation is in accordance with the report of Ogunbanwo*et al.* (2004). They reported that LAB produced the antimicrobial bacteriocin against some pathogens.

Furthermore, the antimicrobial susceptibility test of LAB isolates against food- borne pathogen (*S.aureus*) showed that *L.plantarum* had the highest zone of inhibition. This may be attributed to the low pH in which the organisms grow.This agrees with the work of Fernandez et al. (2001) and Ogunbanwo et al. (2004) who reported that Lactic acid bacteria exert a strong antagonistic activity against food contaminating microorganisms (Spoilage and Pathogenic). Moreover, the antimicrobial susceptibility test against wound-borne pathogen (P. *aeruginosa*) showed that *L.brevis* gave the highest zones of inhibition against*Pseudomonas aeruginosa*. The antibacterial activity of these organisms may be due to the production of lactic acid that lowered the pH of the medium or competition for nutrients or antibacterial compounds. This agree with earlier reports by worker like Bezkorvainy (2001) and Tambeka*et al.* (2009) on the nature of antimicrobial compounds produced by LAB e.g. Lactic acid and their relationship with lowered pH. Additionally, the inhibitory ability of LAB could be due to the presence of various types and concentrations of antimicrobial substances, such as bacteriocins and organic acids (Savadogo*et al.*, 2006). This research however agrees with their report.

SUMMARY

Lactic acid bacteria can produce different metabolites having antimicrobial and probiotic activities in freshly harvested

honey. However, the production of these antibacterial agents can be influenced by biological (the type of starter organism used), physical and chemical parameters under different environmental conditions and the time of harvest of the honey. Our result strongly support that the cell free supernatant of lactic acid bacteria isolated from freshly harvested honey and its comb have good antibacterial activity against clinical and standard human pathogens.

Cultivation of these potential isolates under optimum condition can lead to the production of potential antibacterial agents (like lactic acid, hydrogen peroxide and others) having therapeutic, preservative as well as probiotics activity. This approach may help reduce health problems attributed to consumption of foods contaminated with human pathogens (Adeyemo and Onilude, 2013 and 2014). Therefore, isolation and characterization of novel strains of lactic acid bacteria from freshly harvested honey may lead the pathway to discovery of novel antibacterials for the control and treatment of antibiotics resistance infectious diseases.

DECLARATION OF CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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