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Probiotic Potential Of Lactic Acid Bacteria Isolated From Coconut (Cocos Nucifera) Wine (Mnazi) In Kenya

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Abstract: **Lactic acid bacteria (LAB) are useful in human health as probiotics. To achieve this they need to reach the gastro-intestinal tract and remain viable. This study assessed the probiotic potential of lactic acid bacteria strains isolated from fermented coconut wine (*Mnazi*). A total of fifteen strains were screened *in vitro* for acid tolerance, bile tolerance, survival under conditions of simulated GIT passage and their antimicrobial activity against indicator organisms using standard techniques. The results showed that all fifteen strains had high viable counts after three hours at pH 2.5 but showed reduced viability at pH 2.0 where only eight survived. In general, there was better survival of the isolates after exposure to bile when grown at pH 2.5 than pH 2.0. The strains also survived exposure to the simulated stomach duodenum passage (SSDP) for three hours (37%-56%). They showed variable potent antimicrobial activity against indicator organisms. All the strains inhibited *B. subtilis*, three strains were unable to inhibit *E. coli* while two strains were unable to inhibit *S. aureus*. In conclusion, these findings show that the fifteen strains of lactic acid bacteria isolated from *Mnazi* had desirable probiotic properties as they were able to survive simulated stomach duodenum passage as well as inhibit test pathogenic microorganisms.**

Keywords: **Coconut, *Mnazi*, Probiotics, Lactic acid bacteria, Antimicrobial.**

1. INTRODUCTION

Lactic acid bacteria (LAB) are Gram positive, non-spore forming, catalase negative cocci or rods that are anaerobic, microaerophilic or aero-tolerant and they produce lactic acid as a major product from fermentation [1]. They are useful in industry and human health, including preservation of foods and as probiotics [2]. They normally reside in the mouth and intestinal tract where they enhance immune responses [3]. The beneficial bacteria can be incorporated in the diet to have beneficial effects on the gut microflora [4]. They produce antimicrobials during carbon source metabolism and compete with other species by acidifying their environment and by rapidly depleting nutrients [5]. Besides this, some LAB also produce potent antibiotic compounds such as bacteriocins [5]. The current need for biopreservation has renewed the interest in the search for food compatible antimicrobials produced by microorganisms. There are many different types of probiotic cultures which provide various benefits. Lactic acid bacteria commonly used as probiotic microorganisms in food products are strains belonging to the genera *Bifidobacterium, Enterococcus, Lactobacillus, Lactococcus* and *Streptococcus* [4].

Probiotics represent live microorganisms or microbial preparations or metabolites of stabilized microorganisms which confer beneficial effects on host organisms and affect microbial composition with stimulating effects on digestion and immunity of the organisms [6]. They are classified as probiotics as they exert beneficial effects which include reduction of serum cholesterol, aid in lactose digestion , resistance to enteric pathogens, anti-colon cancer effects, small bowel

bacterial overgrowth, allergy and mucosal barrier dysfunction including diarrhoea, constipation and immune responses and exerting anti-mutagenic activities [2], [3], [7], [8], [9].

When assessing the health promoting effects of probiotics, it's important to keep in mind that all probiotic strains are different. Even strains representing the same species usually have different properties. Several attributes and characteristics of probiotic bacteria should be considered before clinical trials are performed [10], [11]. To exhibit their beneficial effects, probiotic bacteria need to reach its destination. Thus it is necessary for it to be tolerant to acid and bile salts [12]. Adherence to gastrointestinal cells is important for successful colonization and therefore beneficial effects will last longer in gastrointestinal tract [13]. The knowledge of antibiotic susceptibility of potential probiotics strains is necessary in curing, although rare – *Lactobacillus* associated infections [14]. Production of enzyme beta-galactosidase in high amounts will aid in lactose digestion [2] while stains that are able to ferment fructooligosaccharides (FOS) may be used in symbiotic products [15], [16].

Coconut wine (*Mnazi*) is an alcoholic beverage which is produced by fermentation of sugary coconut sap. The coconut sap is tapped from palm trees which grow at the coastal region of Kenya. This alcoholic beverage is a product of a mixed alcoholic, lactic and acetic fermentation. First, the sugary sap is fermented to ethanol within 8-12 hours by yeasts and lactic acid bacteria, creating a highly suitable medium for the development of acetic acid bacteria [17].

Lactic acid bacteria isolated from *Mnazi* have not been assessed for their probiotic potentials or their ability to produce bacteriocins and other inhibitory substances that can act as natural antibiotics against pathogenic microorganisms. To benefit the consumer health-wise, a probiotic bacterium has to reach its target site (gut) alive, it should also have good technological properties so that it can be manufactured and added into foods without losing its viability and functionality, or creating unpleasant flavours or textures and it should survive the passage through the upper GIT and be able to function in the gut environment. This study therefore sought to assess and document the probiotic potential of *Lactobacillus ssp*. isolated from *Mnazi.*

II. MATERIALS AND METHODS

2.1 Samples:

A total of 15 *Lactobacillus ssp*. strains previously isolated from *Mnazi* and identified at Animal Food Functions Laboratory in Okayama University, Japan were used in this study. They were characterised by physiological features and identified to species level using API 50 CHL system (BIOMERIEUX SA, France) [17]. Cultures were maintained in MRS broth with sterile 15% glycerol and stored at -18^{0} C in deep freezer.

2.2 Growth media:

All strains were cultured on de Man, Rogosa and Sharpe (MRS, Oxoid) agar plates. The fully grown colonies were stored on plates at 4° C until further use and sub-cultured on a monthly basis. Cell suspension with glycerol as cryoprotectant was stored in Microbank vials (Pro-lab Diagnostics) at -80° C for long term conservation. Other media used in this study were MRS broth, Agar ultrapure, Nutrient agar and broth (Merck).

2.3 Methods:

2.3.1 Determination of acid tolerance:

Acid tolerance was determined according to [18]. Test culture strains were grown for 16h in MRS broth at 37^{0} C. An aliquot of 1ml of the 16h old culture was inoculated into 9ml MRS broth whose pH was adjusted to 2 and 2.5 using 1N HCL. Samples were drawn after 0, 1, 2 and 3h and immediately diluted ten-fold in Peptone water phosphate- buffered pH 7 to eliminate medium acidity. Decimal dilutions of samples were made using maximum recovery diluent and 0.1ml was taken from 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} of the samples and pour plated in duplicate, in 15ml MRS agar. Plates were incubated at 37^{0} C, anaerobically using anaerobic jars containing wet anaerocult sachets for 48h. Viable counts were determined by counting the number of colonies from plates containing 10 to 300 colonies and colony forming units (CFU/ml) determined from the average. Survival rates were calculated according to the following equation:

$$\text{Survival rate}(\%) = \left(\frac{\log \text{CFU N1}}{\log \text{CFU N0}}\right) \times 100\%$$

Where N1 represents the total viable counts of the tested strains after treatment, N0 represents the total viable counts before treatment.

2.3.2 Determination of bile tolerance:

Bile tolerance was determined according to modified methods of [18]. Following 3h of exposure to pH 2 and pH 2.5, 1 ml of the acid stressed cultures were diluted in peptone water phosphate buffered at pH 7 to eliminate the low pH medium acidity. Aliquots of 1 ml of each of the cultures were then transferred into 9ml MRS broth supplemented with 0.3% bile salts and incubated for 48h at 37^0 C. Viable cell counts were determined after 0, 3, 24 and 48h using pour plate method in MRS agar and incubated at 37^0 C in anaerobic jars containing wet anaerocult sachets. The survival rate was calculated according to the equation above.

2.3.3 Response to Simulated Stomach Duodenum Passage:

Tolerance to passage in the upper gastrointestinal tract (GIT) was assessed according to a method by [19]. The strains were inoculated with bile and artificial duodenum secretions. Sterile MRS broth at pH adjusted to 3.0 with 5 M HCl was used. Synthetic duodenum juice (6.4 g $L^{-1}NaHCO_3$, 0.239 g L^{-1}KCl, 1.28 g L^{-1}NaCl) was prepared in bidest water. The pH was adjusted to 7.4 with 5M HCl before sterilizing at 121^0 C for 15 minutes. The oxgall solution was prepared by reconstituting 10g of oxgall in 100ml bidest water and sterilizing at 121^0 C for 15 minutes. Required volumes of the overnight cultures and MRS broth adjusted to pH 3.0 were aseptically mixed in sterile flasks to give a final concentration of 2×10^8 CFU per 10 ml MRS. After mixing, the initial count was determined by spread plating. The flasks were incubated at 37^0 C. Samples were withdrawn after 1 hour and viable counts determined by spread plating. Four (4) millilitres of oxgal solution were added to the culture in the flasks, followed by 17ml of duodenum juice. After mixing, the flasks were further incubated at 37^0 C. Samples were withdrawn after 2 and 3h, and counts determined as described above. The survival rate was also calculated according to the equation above.

2.3.4 Production of Antimicrobials:

The antimicrobial activity of the tested strains was determined using agar spot method described by [20], [21]. Bacterial pathogens namely gram positive stains *Staphylococcus aureus* ATTC 25923, *Bacillus subtilis* (a gram positive non-lactic acid bacteria strain) and *E.coli* NCTC 10418 (a gram negative bacteria) were used as the indicator organisms. Test isolates were grown anaerobically in MRS broth (to prevent formation of H_20_2) for 24h at 37° C. Cultures were centrifuged at 4193 r.p.m at 4°C for 20 min to obtain a cell free supernatant. Three quarters of the supernatant were neutralized using 1N NaOH to exclude antimicrobial effect of organic acid, then filtered through a 0.2 µm pore size filter.

Nutrient agar seeded with overnight cultures of the indicator strains of *Enterococcus faecalis* NCTC 775, *E.coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 each strain separately, was pour plated and 10mm wells made into the agar. The wells were sealed at the bottom with sterile non-seeded agar. About 100 µl aliquots of sterile neutral supernatant was placed into the agar wells and in duplicates for each test isolate. The plates were kept at 4°C for 2h (to allow for diffusion of antimicrobial substances) then incubated for 48h at the optimum temperature for indicator microorganisms (37° C). The diameter of the inhibition zone surrounding the well was then measured.

2.4 Data analysis:

The data obtained were expressed as means of duplicate experiments. All statistical analysis was performed using SPSS ver. 17.0 for Windows software (SPSS, Inc).

III. RESULTS AND DISCUSSION

Acid tolerance:

To determine acid tolerance, the 15 *Lactobacillus* strains from *Mnazi* were inoculated into sterile MRS broth at pH 2 and 2.5, incubated and their viable counts checked every hour for 3 hours. It is desirable that probiotic microorganisms are able to reach the GIT and remain viable there for 4 hours or more.

All the tested isolates had residual microbial counts greater than 10^7 CFU/ml after 3h of incubation under pH 2.5. Majority of them had a survival rate of 77-90% suggesting they are able to tolerate well stomach conditions. However, only 8 strains (*L. paracasei* TB402, CM203, CM301, TB302, CM4091, CB303, CB4041 and CM201) were able to survive at pH 2 with viable counts of between 10^3-10^6 CFU/ml, the rest had a reduction in their count for the first 1-2h

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but had died by the 3h period (Table 1). In addition, the strains *L. paracasei* CB204, TD3051, CM4081, TB405 CB3021, TM302 and *L. plantarum* CM402 could not tolerate pH 2 but were able to remain viable at pH 2.5 for the 3h period. These results indicate that in general the strains were stable at pH 2.5 but could not be able to grow. They concur with those of [22], who found that *Lactobacilli* strains remained viable after exposure to pH of 2.5-4.0. This can be attributed to the ability of *Lactobacilli* strains to withstand stressful conditions and survive for longer periods in highly acidic environments. A similar study by [18] reported stability but no growth of strains of *Lactobacillus* isolated from *koko* fermented millet porridge) where the isolates were capable of surviving at a level of 10^5 CFU/ml.

Bile Tolerance:

To determine bile tolerance, the 15 *Lactobacillus* strains from *Mnazi* were exposed to pH 2.0 and pH 2.5 at 37° C for 3h and assayed for bile tolerance for up to 48h in MRS broth supplemented with 0.3% bile. In general, there was better survival of the isolates after exposure to bile when grown at pH 2.5 than pH 2.0 (Table 2). At pH 2.5, thirteen (13) of the 15 isolates showed viable counts of 10^6-10^7 CFU/ml, with the lowest being *L .paracasei* TD3051 with a viable count of 10^5 CFU/ml and *L. paracasei* CM4091 showed the highest viable count of 10^9 CFU/ml. Among all the isolates, only *L. paracasei* CB303 and *L .paracasei* CB4041 tolerated growth at pH 2 and exposure to bile. Their viable counts increased by 165% and 190% respectively after the 48h period showing they could tolerate and grow well at 0.3% bile salt, a concentration that mimics the gastrointestinal tract (GIT). The other strains showed no viable count after 3h of exposure (Table 2). These results suggest that these strains are not able to tolerate and grow in conditions containing bile salts and at pH 2.

At pH 2.5, all the isolates except *L. paracasei* CB204 and TB405 were able to grow for up to 48h after exposure to 0.3% bile. The two isolates initially had a viable count of 7.58 and 6.83 log CFU ml^{-1} respectively at 0h after bile exposure but died thereafter. The best growth was registered by the isolates *L. paracasei* TB402, TD3051 and CM201 had viable counts of 9.04, 6.32 and 9.15 log CFU ml^{-1} (survival rates of 117%, 113% and 116%) respectively after 48h of growth. The results suggest that the isolates *L. paracasei* TB402, TD3051 and CM201 from *Mnazi* can tolerate conditions of 0.3% bile and pH 2.5 to grow exponentially. They are in agreement with those from similar studies where *Lactobacilli* strains were viable even after being exposed to bile range of 0.3-0.5% but showed diminished viability at higher bile concentrations [22], [23], [24].

Bacterial response to simulated stomach duodenum passage:

The LAB isolates from *Mnazi* were tested under conditions of simulated stomach duodenum passage to determine their survival. All the isolates showed survival rates of 33-55% after 3h despite the high bile concentration of more than 2% (Table 3). The strains *L. paracasei* TB402, CM203, TB302, CB3021 and CM201 had survival rates of more than 50% while the rest; *L. paracasei* CB204, TD3051, CM4081, CM301, TB405, CM4091, CB303, CB4041, TM302 and *L. plantarum* CM402 had survival rates of less than 50%. This suggests that they can tolerate the conditions of the stomach and can therefore be potent probiotics. Survival at pH 3.0 is significant as ingestion with food raises the pH in stomach to 3.0 or higher [25].

Production of antimicrobial products by isolated stains:

The LAB isolates from *Mnazi* were tested for antimicrobial activity against indicator organisms (Gram negative; *Escherichia coli*, *Enterococcus feacalis* and Gram positive; *Staphylococcus aureus, Bacillus subtilis*). The cell-free supernatants from the different *Lactobacillus* strains inhibited the growth of indicator organisms as shown by inhibition zone results (Table 4 and Fig.1). Among the test strains isolate CB4041 showed the highest antibacterial activity against *E. coli* (18mm), *B. subtilis* (17mm) and *E. feacalis* (18mm) while little activity was observed on isolate CM301 against *B. subtilis* (12mm).

Four (4) strains CB4041, CM4091, CM4081 TB302 and CM203 exhibited moderate potency against gram positive bacteria *S. aureus and B. subtilis.* The strains CB4041, TB302, CM203, and CB303 showed higher potency against gram negative than the gram positive indicator organisms. A lower activity was detected against *E.coli*, *B. subtilis, and S. aureus* in CB3021, TD3051, CM301, TB402, TM302, CM4081, and CM201 demonstrating weak antimicrobial activity against the 4 indicator organisms while strain CM203 showed more resistance to *E. fecalis.*

The inhibition activity of these strains can be attributed to their production of bio-substances with bactericidal or bacteriostatic activities, such as bacteriocin, organic acids, and low molecular weight peptides that are inhibitory to the

pathogens [26], [27]. The gram positive pathogenic bacteria were the most sensitive to the bacteriocin produced by the lactic acid bacteria. The resistance of gram negative bacteria can be attributed to the particular nature of their cellular envelop. This suggests that they can be potent antimicrobial agents against gram negative bacteria which possess strongly defended structure of cell membrane impermeable to several antimicrobial agents resulting to less sensitive to many drugs.

IV. CONCLUSION AND RECOMMENDATION

The present study has shown that the fifteen strains of lactic acid bacteria isolated from *Mnazi* had desirable probiotic properties as they were tolerant to acid, bile, able to survive simulated stomach duodenum passage as well as inhibit test pathogenic microorganisms. Further *in vivo* studies should be carried out using cell lines and animal models with a view of developing a consumer product that can benefit people.

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APPENDIX - A

TABLES AND FIGURES:

Table 1: Survival of Lactobacillus paracasei and Lactobacillus plantarum stains (Log_{10} CFU/ml) isolated from Mnazi under pH 2.0 and 2.5 for up to 3h in MRS broth.

Strain Code	pH 2.0				pH 2.5			
	0h	1h	2h	3h	0h	1h	2h	3h
***L. paracasei* TB402**	9.78	3.70 (37)	7.11 (73)	3.69 (38)	10.91	9.65 (88)	9.11 (83)	8.94 (82)
***L. paracasei* CM203**	9.41	4.04 (43)	5.60 (60)	5.90 (63)	11.70	8.84 (76)	8.68 (74)	8.86 (76)
***L. paracasei* CB204**	11.74	6.20 (53)	5.72 (49)	___	10.65	9.26 (87)	8.62 (81)	8.41 (79)
***L. paracasei* TD3051**	9.36	4.38 (47)	3.30 (35)	___	11.20	9.86 (88)	9.78 (87)	8.26 (74)
***L. plantarum* CM402**	9.74	4.70 (48)	6.40 (66)	___	11.39	8.89 (78)	9.08 (80)	9.45 (83)
***L. paracasei* CM4081**	8.90	4.32 (49)	3.48 (39)	___	10.08	9.52 (94)	9.97 (99)	9.96 (99)
***L. paracasei* CM301**	11.43	4.00 (35)	5.32 (47)	6.11 (53)	10.15	9.18 (90)	9.34 (92)	9.04 (89)
***L. paracasei* TB302**	9.60	6.20 (65)	6.66 (69)	3.60 (37)	11.86	10.04 (85)	8.89 (75)	7.35 (62)
***L. paracasei* TB405**	10.87	8.00 (74)	7.30 (67)	___	12.30	10.25 (83)	9.90 (80)	9.20 (75)
***L. paracasei* CB3021**	10.00	4.85 (49)	___	___	12.26	10.04 (82)	9.18 (75)	8.52 (69)

L. paracasei **CM4091**	11.70	6.38 (54)	4.30 (37)	3.00 (26)	13.64	9.43 (69)	9.11 (67)	8.57 (63)
L. paracasei **CB303**	10.11	3.60 (36)	4.71 (47)	6.28 (62)	12.28	10.00 (81)	9.49 (77)	9.43 (77)
L. paracasei **CB4041**	8.30	4.32 (52)	5.78 (70)	6.15 (74)	11.70	10.30 (88)	10.20 (87)	10.20 (87)
L. paracasei **TM302**	10.62	3.00 (28)	6.80 (64)	___	12.04	9.52 (79)	9.30 (77)	9.30 (77)
L. paracasei **CM201**	9.81	7.54 (77)	5.60 (57)	3.60 (37)	12.26	10.44 (85)	10.32 (84)	7.35 (59)

Figures in brackets represent the survival rate of each strain

Table 2: Survival of Lactobacillus paracasei and Lactobacillus plantarum stains (Log_{10} CFU/ml) isolated from Mnazi in MRS broth supplemented with 0.3% bile salts, following a 3h to 48h exposure to pH 2 and 2.5.

Strain Code	**pH 2.0**				**pH 2.5**			
	0h	**3h**	**24h**	**48h**	**0h**	**3h**	**24h**	**48h**
L. paracasei **TB402**	___	___	___	___	7.70	6.88 (89)	8.79 (114)	9.04 (117)
L. paracasei **CM203**	3.30	___	___	___	7.65	5.78 (76)	4.91 (64)	4.60 (60)
L. paracasei **CB204**	___	___	___	___	7.58	___	___	___
L. paracasei **TD3051**	3.48	___	___	___	5.57	5.85 (105)	6.00 (108)	6.32 (113)
L. plantarum **CM402**	___	___	___	___	7.67	7.32 (95)	7.18 (94)	7.30 (95)
L. paracasei **CM4081**	___	___	___	___	7.65	3.00 (39)	3.78 (49)	6.49 (85)
L. paracasei **CM301**	___	___	___	___	7.68	3.85 (50)	4.30 (56)	6.51 (85)
L. paracasei **TB302**	___	___	___	___	7.89	4.73 (60)	5.15 (65)	6.59 (84)
L. paracasei **TB405**	___	___	___	___	6.83	___	___	___
L. paracasei **CB3021**	4.00	3.00 (75)	___	___	6.62	5.48 (83)	4.79 (72)	4.51 (68)
L.paracasei **CM4091**	3.60	___	___	___	9.45	6.62 (70)	5.95 (63)	5.38 (57)
L.paracasei **CB303**	3.00	3.00 (100)	4.69 (156)	4.96 (165)	7.73	3.85 (50)	3.30 (43)	6.52 (84)
L.paracasei **CB4041**	3.48	4.55 (131)	4.60 (153)	6.60 (190)	7.62	3.34 (44)	4.60 (60)	6.70 (88)
L.paracasei **TM302**	3.00	___	___	___	7.66	4.70 (61)	3.30 (43)	3.48 (45)
L.paracasei **CM201**	4.48	___	___	___	7.92	3.79 (48)	4.73 (60)	9.15 (116)

Table 3: Survival rates of L paracasei and L plantarum strains (Log_{10} CFU/ml) isolated from Mnazi in response to simulated stomach duodenum passage (SSDP), following 3hr exposure at 37°C.

Strain Code	**0h**	**1h**	**2h**	**3h**
L. paracasei **TB402**	10.6	5.56 (52)	5.66 (53)	5.7 (54)
L. paracasei **CM203**	11.3	4.53 (40)	6.23 (55)	6.34 (56)
L. paracasei **CB204**	11.8	4.72 (40)	5.88 (50)	5.75 (49)
L. paracasei **TD3051**	12.1	4.46 (37)	4.95 (41)	5 (41)
L. plantarum **CM402**	12.5	5.7 (46)	5.66 (45)	5.6 (45)
L. paracasei **CM4081**	11.3	4.3 (38)	4.6 (41)	4.48 (40)
L. paracasei **CM301**	11.9	4.28 (36)	4.36 (37)	4.43 (37)
L. paracasei **TB302**	12.4	7.48 (60)	6.66 (54)	6.85 (55)
L. paracasei **TB405**	11.9	4.85 (41)	4.46 (37)	4.41 (37)
L. paracasei **CB3021**	12	7 (58)	6.3 (53)	6.18 (52)
L. paracasei **CM4091**	11.4	5.3 (46)	4.79 (42)	4.79 (42)
L. paracasei **CB303**	11.9	6.52 (55)	5.3 (45)	5.52 (46)
L. paracasei **CB4041**	12.7	5 (39)	4.23 (33)	4.23 (33)
L. paracasei **TM302**	9.78	6.81 (70)	5.93 (61)	3.82 (39)
L. paracasei **CM201**	12.1	7.36 (61)	6.81 (56)	6.72 (56)

Table 4: Antimicrobial effects of the Mnazi bacterial strains on against selected common indicator pathogens (baseline=12mm diameter of the well)

Test Strains	Diameter of the inhibition zones (mm)			
	E. coli	*B. subtilis*	*S. aureus*	*E. feacalis*
L. paracasei CB3021	14	13	13	14
L. paracasei TD 3051	14	14	14	13
L. paracasei CM 301	13	12	13	13
L. paracasei CM 203	13	14	14	17
L. paracasei CB4041	18	17	15	18
L. paracasei TB302	15	14	15	16
L. paracasei TB405	13	15	14	14
L. paracasei TB 402	16	14	13	13
L. paracasei CM4091	14	20	14	15
L. paracasei CB 303	15	14	14	16
L. plantarum CM 402	13	14	13	14
L. paracasei TM302	16	13	13	13
L. paracasei CM4081	13	14	15	14
L. paracasei CM201	15	16	13	14
L. casei (Probiotic)	20	19	18	18

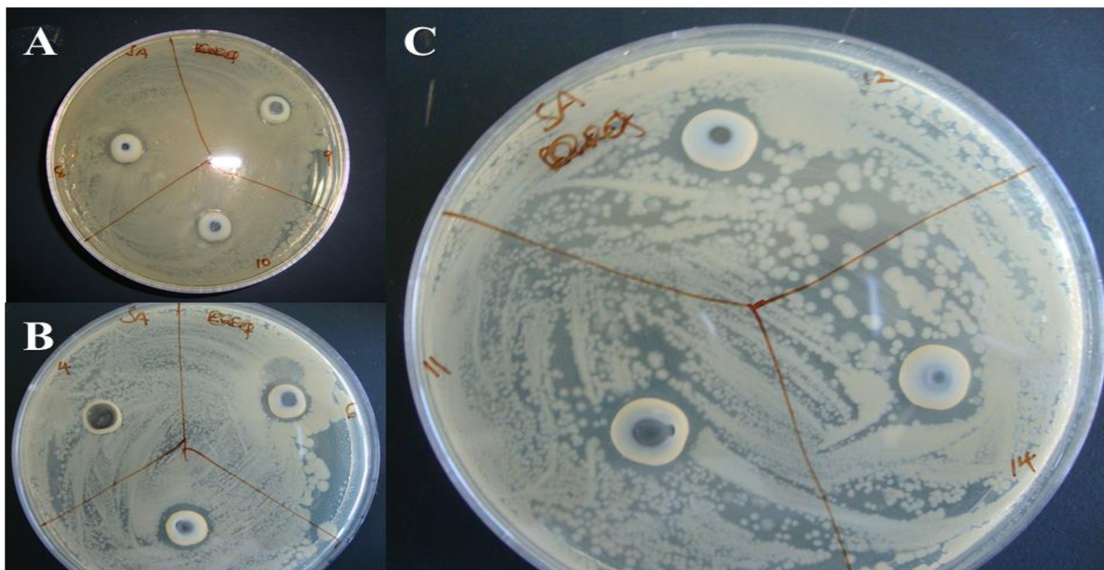

Figure 1: Antimicrobial activity against S. aureus of isolates 8 (TB402), 9 (CM4091), and 10 (CB303) (panel A); 4 (CM203), 6 (TB302) (panel B); 11 (CM402), 12 (TM302), and 14 (CM201) (panel C)