Original Article

The In Vitro Antimicrobial Activities of Metabolites from *Lactobacillus* Strains on *Candida* Species Implicated in *Candida* Vaginitis

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Abstract

***Background*: Research from developing countries, such as Nigeria, on *Lactobacillus* species in the female urogenital tract and their role as a barrier to vaginal infection is limited. Therefore, the aim of this study was to assess the clinical biotherapeutic potential of indigenous *Lactobacillus* species.**

***Methods:* Antimicrobial metabolites production were characterised using simple and easily reproducible qualitative and quantitative methods. The in vitro inhibitory effect of *Lactobacillus* antimicrobials on vulvovaginal candidiasis–associated *Candida* species was investigated using modified agar spot and agar well-diffusion methods.**

***Results:* The maximum levels of lactic acid, hydrogen peroxide, and diacetyl from 20 vaginal *Lactobacillus* strains from diseased subjects were 1.46 mg/L, 1.36 mmol/L, and 1.72 mg/L respectively. From the 4 healthy subjects, the maximum level of lactic acid was 1.08 mg/L; hydrogen peroxide, 1.36 mmol/L; and diacetyl, 0.86 mg/L. The maximum productions of these substances occurred between 72 and 120 hours of incubation. The in vitro antagonistic activities of vaginal *L. acidophilus*, *L. fermentum*, *L. brevis*, *L. plantarum*, *L. casei*, *L. delbrueckii*, and *L. jensenii* from diseased subjects inhibited a maximum of 5.71% of the 35 *Candida* species tested, while vaginal *L. acidophilus* and *L. plantarum* from healthy subjects inhibited between 57.1% and 68.6% of *Candida* species in vitro.**

***Conclusion:* Antimicrobial-producing lactobacilli can be considered as adjunct biotherapeutic candidates for the treatment of vulvovaginal candidiasis.**

Keywords: *antifungal agents, antimicrobial agents,* Candida*, contraceptives,* Lactobacillus*, vulvovaginal candidiasis*

Introduction

The vaginal microflora in its totality is a flexible population that occupies a particular ecological niche and acts as a barrier to the establishment of other microorganisms. Additionally, it has been reported that the vaginal ecosystem contains microbiota that protect it from invading pathogens, including those that are sexually transmissible and those that cause urinary tract infections (1,2). However, the degree of acute stress tolerated by the vaginal microflora must have defined limits. If the acute stress is too extreme, the original microflora may collapse and therefore be unable to provide protection against pathogenic microorganisms that can rapidly proliferate in the environment (3,4), resulting in genitourinary diseases.

Sexually transmissible diseases (STDs) are some of the least recognised health problems worldwide, especially in developing countries like Nigeria (5). In spite of advances in diagnosis and treatment, the number of STD cases has continued to rise and has reached epidemic proportion in many countries. Candidiasis is a mycotic human infection caused by *Candida albicans* and other related pathogenic *Candida* species, and it is one of the most common STDs.

Approximately 3 million or about 75% of women experience vulvovaginal candidiasis (VVC), and almost 10% of them have recurrent VVC (6–8). *Candida* spp. are reported as the most commonly cultured pathogenic microorganisms, and vaginal candidiasis is one of the most common infections seen in general practice (9).

Studies have suggested that the candidal problem is not under control and, in fact, is worsening. Although *Candida* infections are usually treated with an array of antimycotic agents such as azoles, polyenes, echinocandins, allylamines, and other derivatives, the emergence of antimycotic-resistant candidal pathogens, especially the potential widespread dissemination of resistance, has become a major public health concern (10–12). The prevalence of VVC or *Candida* vulvovaginitis is therefore expected to increase. Antifungal resistance has been reported in most *Candida* species, which are the aetiological agents of VVC (5), even in addition to other adverse effects of the drugs. Thus, there is a need for alternative or adjunct bio-antimycotic means of controlling pathogenic *Candida* species that infect humans.

Lactobacilli, which are well known for their potential ability to prevent diseases in humans (13), are also the predominant members of the vaginal flora in healthy women (14–16). Some researchers from developed countries have reported significant in vitro inhibition of pathogenic vaginal *Candida* by certain lactobacilli species isolated from vaginal and non-vaginal sources (1,2,17,18), but similar studies and information from developing countries, such as Nigeria, are very limited. Therefore, the aim of this study was to investigate the in vitro inhibitory effects of *Lactobacillus* antimicrobials from the vaginas of healthy and diseased Nigerian females on *Candida* species associated with human vaginal candidiasis. A comparison of the inhibition profiles of *Candida* species by the antimicrobial metabolites produced by vaginal lactobacilli from healthy and diseased subjects with that of commercial antifungals and contraceptives was also conducted.

Materials and Methods

Strains and culture conditions

The *Candida* strains used were obtained from the original stock of the microbial collections at the Department of Medical Microbiology & Parasitology, University College Hospital, Ibadan, Nigeria. High vaginal swabs and/or endocervical swabs were obtained from female patients aged 3–62 years old who presented at the special treatment clinic of University College Hospital, Ibadan, Nigeria (6). Initial isolation of bacteria from the vaginal specimens were performed on blood agar, chocolate agar, Sabouraud dextrose agar (SDA; LAB M, UK), cysteine lactose electrolyte deficient agar (LAB M, UK), and Chrom agar that were incubated at 32 °C. The *Lactobacillus* strains were obtained from some of the clinical specimens from diseased patients. Control samples were obtained from a control group of 4 healthy subjects, who were 29–35 years of age, had had 4 previous regular menstrual flows, and who were not on antibiotic or antifungal therapy 6 to 12 months prior to collection of the specimens.

The *Lactobacillus* strains were cultured on deMann, Rogosa, and Sharpe (MRS) agar (LAB M, UK) and incubated at 35 °C with 5%–10% CO_2. The purity of the strains was checked, and the pure cultures of the *Lactobacillus* strains were phenotypically identified by classical tests including analysis of cell morphology, homo/heterofermentative and biochemical characteristics, sugar fermentation patterns, and growth at different temperatures. The strains were examined microscopically, and the initial confirmation and identification of the lactobacilli was based on Gram's reaction, catalase reaction using hydrogen peroxide (H_2O_2), growth at 15 °C and 45 °C in MRS medium, gas and acid production from glucose fermentation, and fermentation of lactose, sucrose, arabinose, fructose, and mannitol. The isolates that met the preliminary identification criteria were grown in replicates overnight (18–24 hours) in 10 mL Rogosa broth at 35 °C until the weight of the cell mass was 0.05–0.10 g. Additional taxonomic studies were carried out on the purified isolates based on their biochemical and physiological characteristics (15,19,20). The cells were centrifuged, washed twice in sterile 0.9% NaCl solution, and were stored at 4 °C in Hogness freezing buffer (3.6 mM K_2HPO_4, 1.3 mM KH_2PO_4, 2.0 mM Na-citrate, 1.0 mM $MgSO_4$, 12% glycerol); the cells were then kept frozen. When needed, the frozen *Lactobacillus* strains were allowed to thaw at ambient temperature and then reactivated in MRS broth before sub-culturing on MRS agar. Confirmed pure cultures were sub-cultured in MRS broth until active cultures were obtained for further studies.

Pure *Candida* cultures were identified using a combination of colony morphology on culture media, microscopic morphology, and biochemical characteristics including assimilation of the sugars

cellobiose, dextrose, dulcitol, fructose, galactose, glucose, inositol, lactose, maltose, mannitol, mellibiose, raffinose, rhamnose, saccharose, sorbitol, sucrose, and xylose. In addition, fresh wet mount examinations (wet preparations) and germinal tube assays were also performed on the strains, and the final identification was made according to the methods of Kreger-van Rij (21). Pure cultures of the *Candida* strains were stored as *Candida* stock strains at 12 °C on SDA slants containing 0.25 mg streptomycin. The strains were later reactivated in SDA broth and then subcultured by streaking on SDA agar to obtain viable, pure, fresh cultures.

Qualitative determination of antimicrobial metabolites produced by the Lactobacillus *strains using antimicrobial assay*

The detection of antagonistic activity by the *Lactobacillus* strains was performed by the modification of agar spot and agar well-diffusion methods (22) of Tagg et al. (23). Wells of 6.0-mm diameter were bored into sterile SDA plates, and 500 µL of each *Candida* strain was seeded on the sterile agar plates. The plates were then left at ambient temperature under aseptic condition for 30 minutes before 250–1000 µL of each 24- to 36-hour-old *Lactobacillus* strain, in MRS semi-solid agar (5% agar), was added to the agar wells and then directly onto another set of pre-seeded SDA agar plates, as indicated in the agar spot diffusion protocol. The plates were incubated at 35 °C for 24–48 hours, after which the zones of inhibition were measured and the diameter (in mm) was recorded. A zone of inhibition of less than 10.0 mm or an absence of a zone of inhibition were recorded as resistant (negative).

In vitro antimycotic susceptibility testing using antifungal agents

The in vitro susceptibility/resistance of *Candida* strains to antimycotic agents commonly available in Nigeria, namely Diflucan capsules, doxycycline capsules, Fungoral tablets, Mycoten tablets/cream, Canesten tablets/cream (clotrimazole), Tetradox (doxycycline), Mycostatin (nystatin), and Flagyl, was determined using SDA agar plates after 24 and 48 hours of incubation at 35 °C as per the modified agar well-diffusion method (22) of Tagg et al. (23). Wells of 6.0-mm diameter were bored into sterile SDA plates and seeded with 500 µL of each *Candida* strain. The plates were incubated at ambient temperature under aseptic condition for 30 minutes, and afterwards 1 mL of each of the antifungal agents, dissolved in plain, sterile semi-solid agar (5%) at 45 °C, was dispensed into the wells. The plates were incubated uninverted at 35 °C for 24–48 hours, after which the diameters (in mm) of the zones of inhibition were measured and recorded. A zone of inhibition less than 10.0 mm or the absence of a zone of inhibition was recorded as resistant (negative). The concentrations of the *Candida* isolates in the inoculum suspensions used in the test were between 1.6 and 2.4 × 10^3 cells/mL.

In vitro antimycotic susceptibility testing using contraceptives

The modified agar well diffusion method (22) of Tagg et al. (23) was used to determine the in vitro susceptibility/resistance of *Candida* strains to commonly available contraceptives in Nigeria: Confidence (Duofem) tablets (ferrous fumarate, brown), Confidence (Duofem) tablets (ferrous fumarate, white), Norquest Fe tablets (norethindrone/ethinyl oestradiol/ferrous fumarate, green), Norquest Fe tablets (norethindrone/ethinyl oestradiol/ferrous fumarate, yellow), and Ovrette tablets (norgestrel). Wells of 6.0-mm diameter were bored into sterile SDA plates and then seeded with 500 µL of each *Candida* strain. The plates were incubated at ambient temperature under aseptic condition for 30 minutes and then 1 mL of each of the contraceptives, dissolved in plain semi-solid agar (5% agar), was dispensed into the wells. The plates were incubated uninverted at 35 °C for 24–48 hours, after which, the zones of inhibition were measured, and the diameters (in mm) were recorded. A zone of inhibition less than 10.0 mm in diameter or the absence of a zone of inhibition was recorded as resistant (negative). The concentrations of the *Candida* inoculum suspensions used in the test were between 1.6 and 2.4 × 10^3 cells/mL.

Quantitative production of antimicrobial metabolites by lactobacilli species

The stock *Lactobacillus* strains were grown in 10-mL Rogosa broth at 35 °C for 48 hours. When the weight of the cell mass reached between 0.05–0.10 g, 10 mL of each fresh *Lactobacillus* culture was re-suspended in MRS broth and incubated for 24–20 hours at 35 °C without agitation. The culture medium was sampled every 24 hours and centrifuged at 3000 rpm for 15 minutes; the supernatants were then used to test for antimicrobial metabolite production.

Quantitative determination of lactic acid

Production of lactic acid by the *Lactobacillus* strains was determined by the Association of Analytical Communities (AOAC)'s method (24). The supernatant from the MRS broth culture of each test organism was titrated with freshly prepared 0.25 M NaOH and 1 mL of phenolphthalein indicator (0.5% w/v in 50% ethanol). The titratable acidity was calculated as percentage of lactic acid using the following equation:

$$\text{Titratable acidity} = \frac{V_{NaOH} \times N_{NaOH} \times ME}{V_{sample}} \times 100$$

where V = volume (mL), N = normality, and ME = equivalence factor. Each millilitre of 1 N NaOH is equivalent to 90.08 mg of lactic acid.

Quantitative determination of H_2O_2

The H_2O_2 produced by the *Lactobacillus* strains was determined according to the AOAC's method (24). First, 25 mL of supernatant from the MRS broth culture of each test organism was transferred to a 150-mL conical flask. Next, 25 mL of freshly prepared sulphuric acid (H_2SO_4) was added, followed by titration with 0.1 N potassium permanganate ($KMnO_4$). Decolourisation of the sample was regarded as the end point. The H_2O_2 production was calculated as follows:

$$H_2O_2 \text{ production} = \frac{V_{KMnO4} \times N_{KMnO4} \times ME}{V_{H2SO4} \times V_{sample}} \times 100$$

where V = volume (mL), N = normality, and ME = equivalence factor. Each millilitre of 0.1 N $KMnO_4$ is equivalent to 1.701 mg of H_2O_2.

Quantitative determination of diacetyl production

To determine the concentration of diacetyl produced by the *Lactobacillus* strains, we used the method from the Food Chemicals Codex (25). Firstly, 25 mL of the supernatant from the MRS broth culture of each test organism was transferred into a 150-mL conical flask, and 7.5 mL of freshly prepared 1 M hydroxylamine solution was added. The same quantity of hydroxylamine solution was dispensed into another 150-mL conical flask for residual titration. Three drops of bromophenol blue indicator was added to each flask. Both flasks were then titrated with 0.1 N HCl to a greenish-yellow end point. The diacetyl concentration was calculated using the following formula:

$$\text{Diacetyl production} = \frac{(V_a - V_s) \times ME}{V_w} \times 100$$

where V_a = volume (mL) of 0.1 N HCl consumed during the titration, V_s = volume (mL) of 0.1 N HCl consumed in the residual titration, ME = equivalence factor, and V_w = volume (mL) of broth culture used during the titration. The equivalence factor of HCl to diacetyl is 21.52 mg.

Results

Out of the 20 *Lactobacillus* strains that were isolated from the endocervical and high vaginal swabs of diseased patients, 5 were identified as *L. acidophilus* (VL1, VL2, VL3, VL4, VL5), 5 as *L. fermentum* (VL6, VL7, VL8, VL9, VL10), 3 as L. brevis (VL11, VL12, VL13), 2 as *L. plantarum* (VL14, VL15), 2 as *L. casei* (VL16, VL17), 2 as *L. delbrueckii* (VL18, VL19), and 1 as *L. jensenii* (VL20). Out of the 4 strains isolated from the healthy subjects, 2 were identified as *L. acidophilus* (VLHS1, VLHS2) and the other 2 as *L. plantarum* (VLHS3, VLHS4).

As shown in Table 1, the in vitro antagonistic activity of the cell-free supernatant of cultures from the diseased patients' vaginal *Lactobacillus* strains gave an overall maximum inhibition rate of 5.71% against *Candida* species. The zones of inhibition were between minimal (10.0–18.0 mm in diameter) and moderate (20.0–25.0 mm in diameter) susceptibility. However, the healthy subjects' vaginal *Lactobacillus* strains had in vitro inhibition rates of between 57.1% and 68.6% against the *Candida* species (Table 2). The recorded zones of inhibition were between 10.0 and 28.0 mm in diameter, but most of the inhibitory activity was also between minimal and moderate susceptibility. The inhibitory effects of the tested antifungals were more prominent, with inhibition rates ranging 57.7%–92.3% (Table 3). Similarly, most of the inhibitory activities (10.0–35.0 mm in diameter) were either moderate or maximal. No inhibition of the *Candida* strains by the contraceptives was recorded.

There was no consistent pattern in the production of antimicrobial metabolites (lactic acid, hydrogen peroxide, and diacetyl) by the vaginal *Lactobacillus* strains from diseased patients. The maximum antimicrobial metabolite production was between 72 and 120 hours of incubation (Figures 1–3). Similar values of antimicrobial production were also recorded for the vaginal *Lactobacillus* strains from

Table 1: The antimicrobial activities of the cell-free supernatants from the vaginal Lactobacillus strains of diseased patients on the Candida strains

Candida strain	Antimicrobial-producing *Lactobacillus* strain [a]						
	VL2	VL7	VL10	VL14	VL16	VL19	Others
C. albicans							
- 6C1	R	R	R	R	R	20.0	R
- AC1, AC2, BC1,FC1, FC2, GC1, GC2, X1C, IC,2C2, 4C2, H2C	R	R	R	R	R	R	R
C. glabrata							
- BC1, 1C2, X7C, 4C1, 1TC, HC	R	R	R	R	R	R	R
C. pseudotropicalis							
- 2C1	R	R	19.0	R	R	15.0	R
- 6C2	R	25.0	R	R	20.0	R	R
- 2C2B, X7C, 9C2	R	R	R	R	R	R	R
C. tropicalis							
- 9C	10.0	18.0	R	R	R	R	R
- 10C	R	R	R	20.0	R	R	R
- HC, 2TC, 6C, ITC2, 2TC2, 6C1A, HC3, HC1, 9CB	R	R	R	R	R	R	R

R indicates absence of zone of inhibition (resistance).

[a] VL1–5 = *L. acidophilus*, VL6–10 = *L. fermentum*, VL11–13 = *L. brevis*, VL14–15 = *L. plantarum*, VL16–17 = *L. casei*, VL18–19 = *L. delbrueckii*, VL20 = *L. jensenii*.

healthy subjects (VLHS1 = *L. acidophilus 1*, VLHS2 = *L. acidophilus 2*, VLHS3 = *L. plantarum 3*, VLHS4 = *L. plantarum 4*). The maximum production rates of lactic acid, H_2O_2, and diacetyl by the *Lactobacillus* strains from diseased patients were 1.46 mg/L, 1.36 mmol/L, and 1.72 mg/L, while those from the healthy subjects were 1.08 mg/L, 1.36 mmol/L, and 0.86 mg/L, respectively (Figures 1–3).

Discussion

VVC, which presents with common symptoms like considerable itching and offensive vaginal discharge (26), and sometimes a burning sensation, is a common infection that affects the quality of life for many women. Generally, the affected women will turn to self-medication with over-the-counter antifungal drugs (e.g., imidazoles, polyenes, and ketoconazoles), which are used as either topical or systemic antifungal agents (6,27). Meanwhile, pathogenic *Candida* species have developed resistance to several antifungal agents (28,29), and another potential limitation of the antifungal drugs is the frequency of their interactions with co-administered drugs, which sometimes results in adverse clinical consequences (30).

The vaginal microflora of healthy asymptomatic women is dominated by diverse species of anaerobic, aerobic, microaerophilic, as well as facultative anaerobic lactobacilli flora (1). Reports characterising and selecting strains of *Lactobacillus* for potential use as probiotics for regenerating the vaginal flora of women with recurrent episodes of bacterial vaginosis indicate that the species recovered were *L. acidophilus*, *L. crispatus*, and *L. delbrueckii* ssp. *delbrueckii* (16,31–33). Similar species (*L. acidophilus, L. brevis, L. casei, L. delbrueckii, L. fermentum, L. jensenii,* and *L. plantarum*) were also recovered from vaginal specimens of both healthy subjects and diseased patients in this study.

The role of *Lactobacillus* species in the female urogenital tract as a barrier to infection is of considerable interest (14) because *Lactobacillus* species are believed to contribute to the control of vaginal microbiota by competing with other microflora for adherence to the vaginal epithelial cells and also by producing antimicrobial compounds such as H_2O_2, organic acids, and bacteriocin-like substances

Table 2: The antimicrobial activity of the cell-free supernatant from the vaginal *Lactobacillus* strains of healthy subjects on the *Candida* strains

***Candida* strain**	**Antimicrobial-producing *Lactobacillus* strain [a]**			
	VLHS1	**VLHS2**	**VLHS**	**VLHS4**
C. albicans				
- AC1	12.0	22.0	15.0	12.0
- AC2	15.0	R	18.0	10.0
- BC1	R	18.0	22.0	12.0
- FC1	10.0	R	R	R
- FC2	10.0	10.0	R	12.0
- GC1	R	10.0	15.0	15.0
- X1C	12.0	10.0	15.0	15.0
- IC	12.0	R	15.0	10.0
- 2C2	10.0	12.0	R	R
- 6C1	10.0	12.0	14.0	12.0
- GC2, 4C2, H2C	R	R	R	R
C. glabrata				
- BC1	14.0	10.0	R	R
- 1C2	12.0	10.0	18.0	12.0
- X7C	R	R	16.0	10.0
- 4C1	16.0	10.0	16.0	19.0
- 1TC	R	R	R	12.0
- HC	12.0	10.0	14.0	12.0
C. pseudotropicalis				
- 2C1	10.0	R	R	22.0
- 6C2	R	10.0	28.0	12.0
- 2C2B	10.0	10.0	R	R
- X7C	R	R	14.0	10.0
- 9C2	10.0	12.0	R	R
C. tropicalis				
- HC	18.0	16.0	R	15.0
- 2TC	10.0	12.0	18.0	12.0
- 6C	R	R	R	18.0
- 9C	10.0	12.0	18.0	12.0
- 10C	12.0	14.0	12.0	10.0
- ITC2	12.0	10.0	R	14.0
- 2TC2	12.0	10.0	12.0	R
- 6C1A	R	R	18.0	10.0
- HC3	12.0	10.0	12.0	12.0
- HC1	14.0	R	R	12.0
- 9CB	10.0	10.0	16.0	18.0
Total % susceptibility	**68.6**	**62.9**	**57.1**	**68.6**

R indicates absence of zone of inhibition (resistance).
[a] VLHS1–2 = *L. acidophilus*, VLHS3–4 = *L. plantarum*.

Table 3: Antagonistic activity of the antimycotic agents on the *Candida* strains

***Candida* strain**	**Antimycotic agent [a]**								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
C. albicans									
- IC	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
- 2C2	20.0	R	25.0	25.0	20.0	20.0	20.0	R	20.0
- H2C	30.0	R	R	R	20.0	25.0	22.0	25.0	30.0
- GC1	R	R	30.0	25.0	10.0	35.0	R	10.0	15.0
- FC1	10.0	R	20.0	20.0	R	25.0	R	20.0	20.0
- AC1	10.0	R	30.0	20.0	20.0	30.0	R	20.0	20.0
- AC2	R	10.0	30.0	20.0	10.0	10.0	10.0	10.0	20.0
- BC1	10.0	R	20.0	20.0	10.0	10.0	R	20.0	20.0
- GC2	20.0	10.0	30.0	20.0	20.0	20.0	R	10.0	10.0
- FC2	10.0	10.0	20.0	20.0	20.0	20.0	R	20.0	10.0
- 4C2	R	R	R	R	R	R	R	R	R
- 6C1	10.0	10.0	30.0	10.0	10.0	10.0	10.0	10.0	10.0
C. glabrata									
- 4C1	R	R	25.0	20.0	R	20.0	R	10.0	10.0
- ITC	20.0	20.0	35.0	20.0	20.0	25.0	R	20.0	25.0
- IC2	20.0	10.0	25.0	20.0	10.0	10.0	R	R	R
- HC	10.0	10.0	20.0	20.0	10.0	20.0	10.0	10.0	10.0
- BC1	10.0	20.0	30.0	20.0	10.0	20.0	R	10.0	R
C. pseudotropicalis									
- X7C	10.0	10.0	30.0	20.0	10.0	10.0	R	10.0	10.0
- 6C2	R	R	R	R	R	R	R	R	R
- 2C1	10.0	10.0	30.0	20.0	10.0	15.0	10.0	10.0	10.0
C. tropicalis									
- HC	R	R	30.0	20.0	10.0	20.0	R	10.0	10.0
- *6C*	10.0	10.0	30.0	20.0	10.0	20.0	R	10.0	10.0
- 9C	R	R	25.0	20.0	10.0	10.0	10.0	15.0	10.0
- 10C	10.0	10.0	20.0	20.0	10.0	20.0	R	20.0	10.0
- 2TC	10.0	10.0	20.0	20.0	10.0	10.0	R	10.0	R
- HC1	R	10.0	20.0	10.0	10.0	R	10.0	10.0	10.0
Total % susceptibility	**69.2**	**57.7**	**88.4**	**88.4**	**88.4**	**92.3**	**30.8**	**84.6**	**84.6**

R indicates absence of zone of inhibition (resistance).

[a] 1 = doxycycline capsule, 2 = Mycoten tablet, 3 = Diflucan capsule, 4 = Mycostatin tablet, 5 = Canesten tablet, 6 = Fungoral tablet, 7 = Flagyl tablet, 8 = Canensten cream, 9 = Mycoten cream

(possibly biosurfactants), which lower the vaginal pH (4,16,17,34,35). In this study, we found minimal to moderate inhibition of *Candida* species by antimicrobials (lactic acid, H_2O_2, and diacetyl) produced from *Lactobacillus* species. Lactic acid, one of the inhibitory agents produced by the screened *Lactobacillus* species, is the major end product of the carbohydrate catabolism of lactic acid bacteria. The maximum amount of lactic acid produced by vaginal *Lactobacillus* strains in this study was 27.0 g/L.

H_2O_2, which generates cytotoxic reactive oxygen, superoxide anions, and hydroxyl radicals in the vaginal fluid (36), has been considered

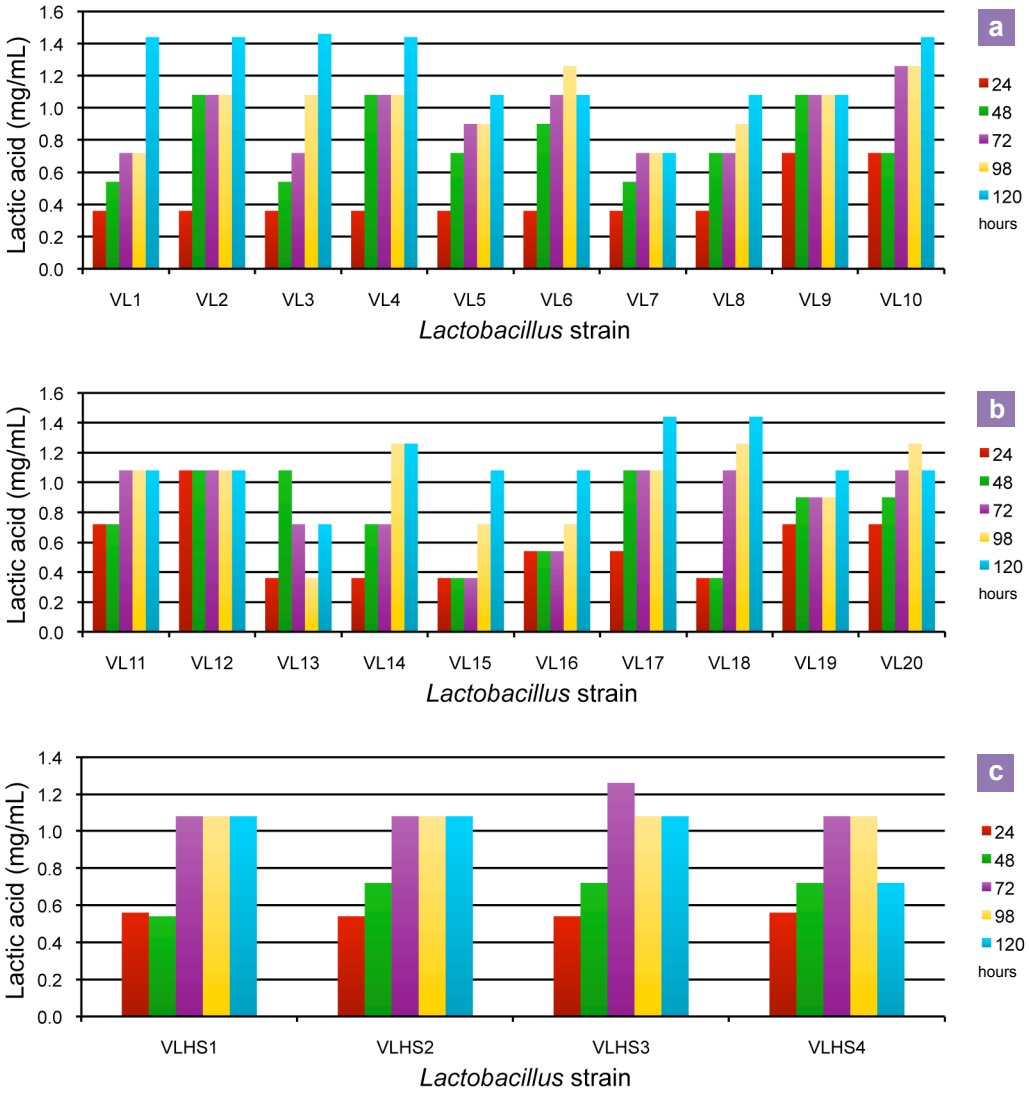

Figure 1: Lactic acid production by *Lactobacillus* species from diseased patients (a: VL1–VL10, b: VL11–VL20) and healthy subjects (c: VLHS1–4) in non-agitated MRS broth.

a key factor in the antagonism of pathogens by *Lactobacillus*. Some studies have noted that *Lactobacillus* species do not have a protective role against vaginal candidiasis per se (16,37,38). However, the metabolic production and release of H_2O_2 by lactic acid bacteria has been reported to be antagonistic towards several other bacteria. Moreover, the accumulated level of H_2O_2 in the culture medium has been found to be auto-inhibitory (39). H_2O_2 inhibits the growth of undesirable microorganisms and may also react with other components to form additional inhibitory compounds. The maximum amount of H_2O_2 produced by the vaginal *Lactobacillus* strains in this study was 1.36 mmol/L, and it was confirmed that the H_2O_2-producing strains were moderately inhibitory against the pathogenic *Candida* species in vitro. Moderate inhibition due to H_2O_2 production has been reported previously (33,40).

In this study, all of the *Lactobacillus* strains produced varying amounts of diacetyl. The maximum amount of diacetyl produced was 0.86 mg/mL, which is consistent with earlier reports, including those of Daeschel (41) and Vandenbergh (42), which found that substances produced by lactic acid bacteria, other than lactic and acetic acids, are produced in much smaller amounts. Diacetyl has also been reported to possess antimicrobial activity (43).

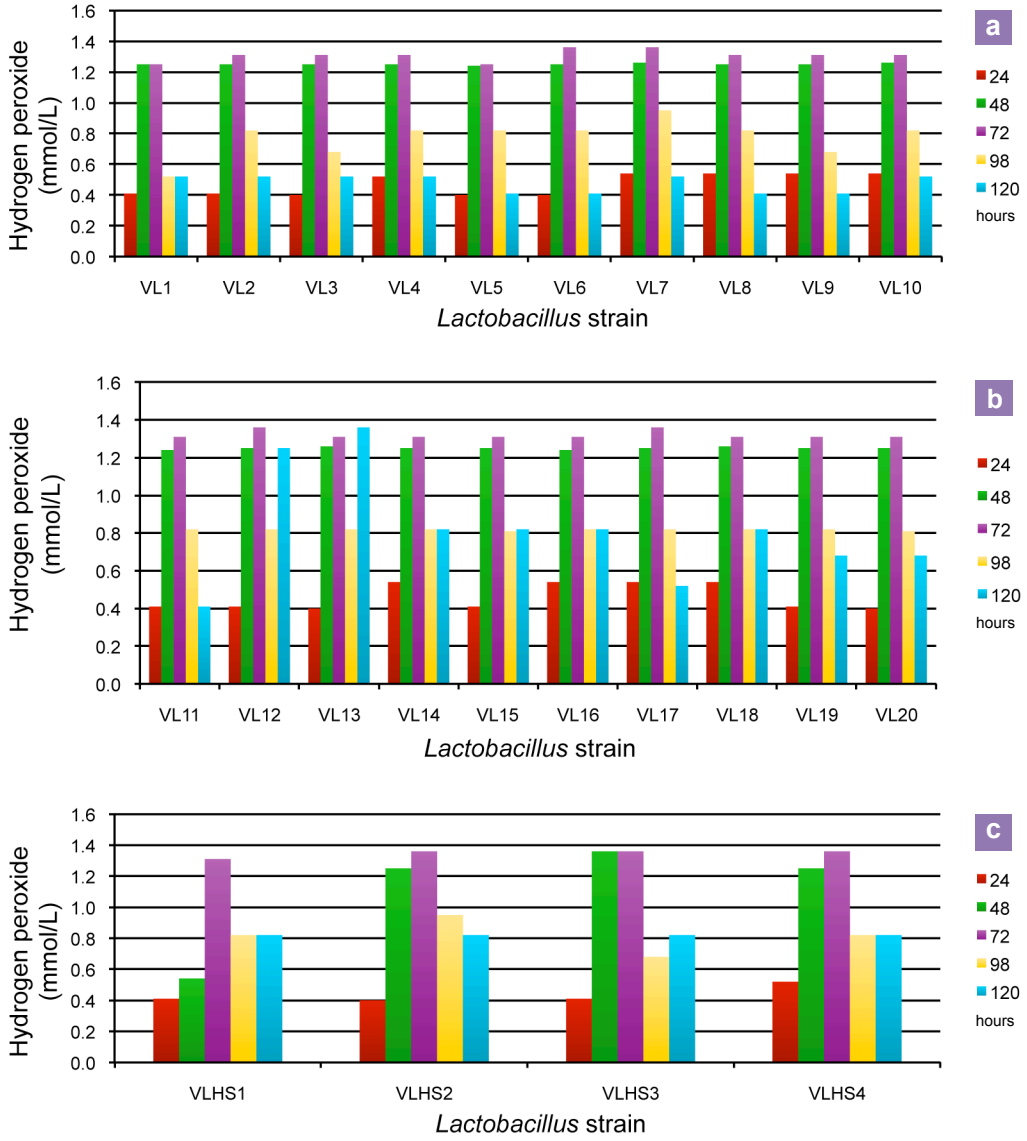

Figure 2: Hydrogen peroxide production by *Lactobacillus* species from diseased patients (a: VL1–VL10, b: VL11–VL20) and healthy subjects (c: VLHS1–4) in non-agitated MRS broth.

The ability of citrate-positive *Lactococcus lactis* ssp. *lactis* (formerly *Streptococcus lactis* ssp. *diacetylactis*) to produce diacetyl and acetoin from citrate has led to its widespread use as a characteristic aroma-producer in many dairy products (44). Although not conclusive, the characteristic aroma produced by lactic acid bacteria, due to diacetyl and acetoin, may be responsible for the better smell of the *Lactobacillus* strains obtained from the healthy subjects as compared with the pungent smell from the diseased patients.

It has been reported that hormonal changes predispose women to vaginal candidiasis (45). It was also confirmed that oral contraceptives may influence the recurrence of symptomatic VVC (46), and according to Maccato et al. (6), women who use high doses of oral contraceptives, contraceptive sponges, and antibiotics are at increased risk of colonisation and symptomatic vaginitis. The results obtained in the study of Fidel et al. (47) suggested that oestrogen, but not progesterone, is an important factor in hormone-associated susceptibility to *C. albicans* vaginitis; the use of oral or injectable hormonal contraception has been found to alter susceptibility to sexually transmitted diseases. Meanwhile, a number of females, especially non-literate women and those

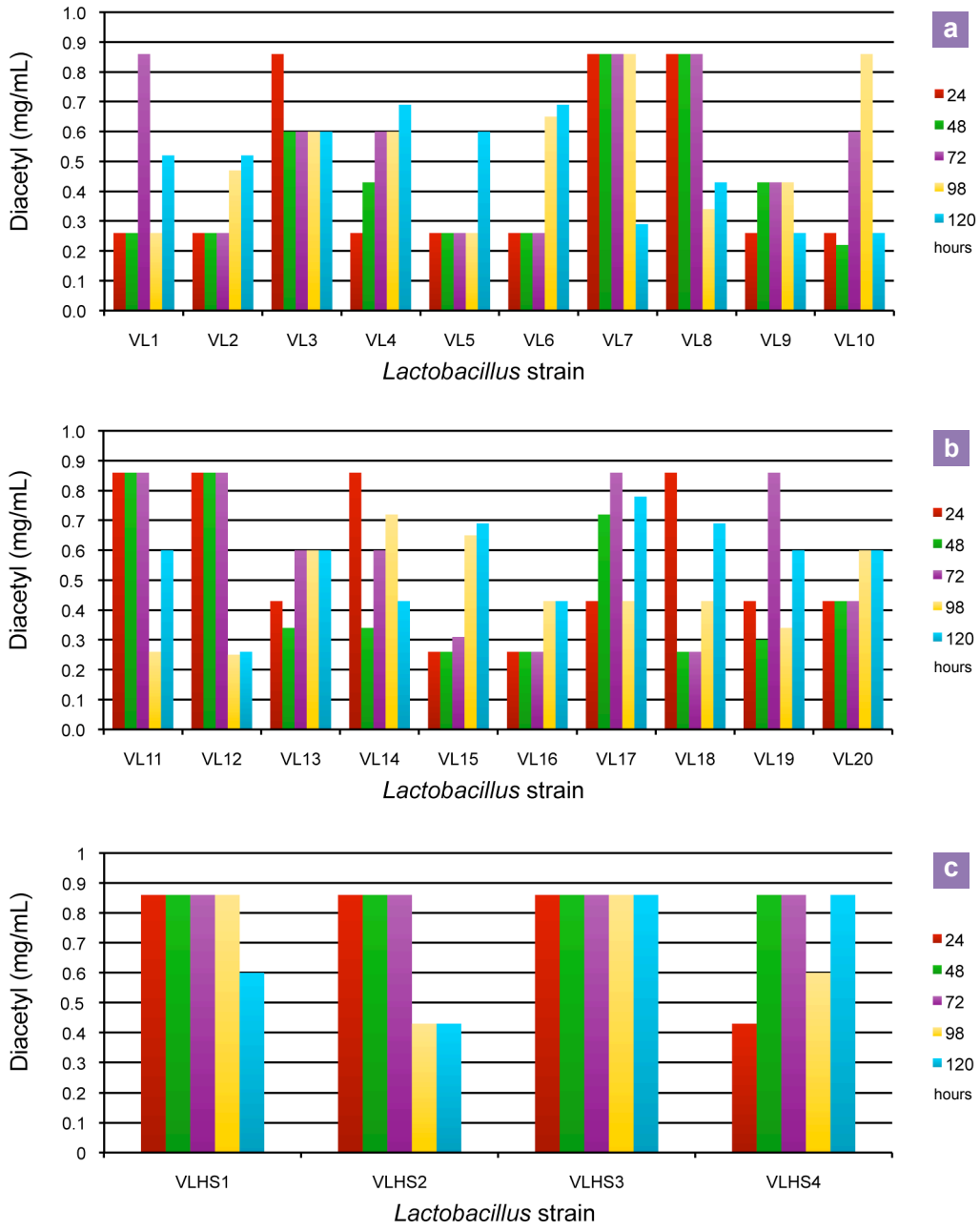

Figure 3: Diacetyl production by *Lactobacillus* species from diseased patients (a: VL1–VL10, b: VL11–VL20) and healthy subjects (c: VLHS1–4) in non-agitated MRS broth.

who work in the sex trade in Nigeria, believe that contraceptives prevent sexually transmitted infections in addition to preventing pregnancy.

Although the role of reproductive hormones in the acquisition of vaginal candidiasis remains unclear (47), a study by Baeten et al. (48) found that users of oral contraceptives were at an increased risk for *Chlamydia* infection and vaginal candidiasis when compared with women who were not using contraception. Oestrogen was found to reduce the ability of vaginal epithelial cells to inhibit the growth of *C. albicans* (47), which is contradictory to the assertion by some women, such as the sexual workers mentioned above, that contraceptives prevent sexually transmitted infections. As reported in this study, none of the most commonly available contraceptives in the country inhibited the *Candida* strains in vitro.

In a review article, Apisarnthanarax et al. (49) even noted that the use of combined oestrogenic oral contraceptives was more commonly associated with candidiasis. The results obtained in the present study therefore confirm that commonly available oral contraceptives in Nigeria have no in vitro inhibitory effect on pathogenic *Candida* strains isolated from clinical cases of candidiasis.

Conclusion

Basic knowledge of the vaginal ecosystem and new research can lead to a successful therapeutic approach (4). The empirical use of inhibitory microorganisms, as highlighted by the findings of this study, indicate that lactobacilli can be considered a potential adjunct bio-therapeutic agent in women with VVC, especially for those with resistant strains or those who have adverse effects or contraindications when using antifungal agents. The inhibitory activities of the vaginal *Lactobacillus* species must have been synergistically enhanced by the production of antimicrobials. The protective role of lactobacilli in preventing VVC is controversial because the recovery of antimicrobial-producing lactobacilli can be dependent on diet and geographical location. Therefore, it is very important that further studies be performed on additional indigenous *Lactobacillus* strains to determine their clinical potential for the adjunct treatment of VVC.

Authors' Contributions

Conception and design, critical revision of the article: AAOO
Provision of study materials: AAOO, MAO
Collection and assembly of data: AAOO, VBB
Analysis and interpretation of the data, drafting and final approval of the article: AAOO, MAO, VBB

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