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Characterisation of the Probiotic Qualities Exhibited by Lactobacilli Strains Isolated from the Anogenital Tract

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ABSTRACT

Introduction: Lactobacilli are well-documented probiotics that exert health benefits on their host. They exhibit characteristics that make them potential alternative treatments to address the antimicrobial resistance conundrum and diseases. Their mechanism of action varies with strain and species. Five lactobacilli strains previously isolated from the anogenital region were subjected to several assessments highlighted in the FAO/WHO document, 'Guidelines for the Evaluation of Probiotics in Food' to determine its suitability as potential probiotics. **Methods:** The five lactobacilli strains were subcultured onto Man de Rogosa agar (MRS). Their ability to auto- and co-aggregate was determined spectrophotometrically. Simultaneously, the cell surface hydrophobic properties of these strains towards xylene and toluene were evaluated using the microbial adhesion to hydrocarbon (MATH) test. The lactobacilli strains were also tested for their ability to withstand acid, bile and spermicide to determine their level of tolerance. **Results:** *Lact. reuteri* 29A, *L. delbrueckii* 45E and *L. reuteri* 29B exhibited the highest degree of auto- and co-aggregation properties. These lactobacilli strains also demonstrated high cell surface hydrophobicity, with the exception of *L. delbrueckii* 45E. Further tests to evaluate the isolated lactobacilli tolerance identified *L. reuteri* 29B as the most tolerant strain towards low pH (pH 2.5 for 4 h), high bile concentration (0.5% for 4 h) and high spermicides concentration (up to 10%). **Conclusion:** Out of the five lactobacilli strains which possessed high antimicrobial activities, *L. reuteri* 29B portrayed the best probiotic qualities with good auto- and co-aggregation abilities and high tolerance against acid, bile and spermicide.

Keywords: Lactobacilli, Probiotic, Anogenital region, *Lactobacillus reuteri*

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INTRODUCTION

Lactobacilli are anae-tolerant, Gram positive bacteria native to the vagina (1, 2) and gut (2, 3). They are well-documented probiotics that exert positive health benefits on the host with fewer or limited adverse effects. This genus has been reported to produce antimicrobial substances that inhibit the proliferation of pathogenic and opportunistic microorganisms. They have also been reported to exhibit other characteristics such as adherence to the host cells and tolerance towards highly acidic environments. Lactobacilli are considered potential alternative treatments to combat the increasing antimicrobial resistance (AMR) because of the extensive data on this genus against multidrug resistant microorganisms. Members of the public are willing to accept probiotics due to their perception that probiotics are a form of natural therapy (4).

Probiotics affect targeted population in ways that is strongly dependent on the strains employed (5). Studies conducted on the lactobacilli isolated from the anogenital region indicate that the effects exhibited are strain- and species-dependent (as reported in our previous study). Due to the differences in mechanism among strains, it is necessary to evaluate them following the guidelines outlined by FAO/WHO in the document, 'Guidelines for the Evaluation of Probiotics in Food' (6) for new probiotic strains.

One of these criteria in the guideline states that lactobacilli of interest should be of human origin, be genetically stable and non-pathogenic. They are to remain viable along the gastrointestinal tract (GIT) by withstanding harsh conditions such as low pH and high bile concentrations (7). Moreover, the probiotic activities of the strains should be tested both in vitro and in vivo to ensure that the effects associated with the strains are reproducible in well-designed human

studies (8). Commercialised lactobacilli strains such as *Lactobacillus reuteri* RC-14 and *Lactobacillus rhamnosus* GR-1 have been identified using these criteria and the guideline is important in the initial screening stages to identify the ideal probiotic strains. The main aim of the present study is to evaluate the probiotic characteristics of the lactobacilli strains previously isolated from the anogenital region of healthy women according to the joint FAO/WHO guidelines (6).

MATERIALS AND METHODS

Culture growth conditions

Five lactobacilli strains labelled as *Lactobacillus delbrueckii* 45E, *Lactobacillus fermentum* 28E, *Lactobacillus mucosae* 28C, *Lactobacillus reuteri* 29A and 29B as well as control strain, *L. reuteri* RC-14 were streaked onto Man de Rogosa, Sharpe agar and incubated at 48 h at 37°C, using AnaeroGen™ 2.5L (Oxoid, UK). Four of the strains were isolated from the posterior fornix whereas, only one strain, *L. reuteri* 29A was isolated from the perianal. The *Candida albicans* strains were cultured using 4% Sabauraud dextrose agar (SDA) (Merck, Germany) and incubated at 37°C for 24 h. All strains were subcultured twice prior to use.

Autoaggregation and coaggregation assay

The autoaggregation abilities of the lactobacilli strains isolated were evaluated using a spectrophotometric autoaggregation assay (9, 10). The 48 h cultures of lactobacilli strains in MRS broth were rinsed twice and resuspended in PBS solution (pH 7.4) at an absorbance of 0.5 ± 0.02 at OD_{600}, which is approximately 1 x 10^7 CFU/mL. The cell suspensions were vortexed for 10 s with a vortex mixer (MixMate, Eppendorf, U.S.A.) and incubated at 37°C, aerobically for 4 and 24 h. The absorbance of the cell suspensions were determined using a spectrophotometer (BioPhotometer plus, Eppendorf, U.S.A.). The autoaggregation ability of each isolate were expressed in percentage (%) using the formula previously described (10), 100 x [1-OD_1/OD_2]; whereby, OD_1 represents the absorbance of the lactobacilli strains at 4 or 24 h and OD_2 represents the absorbance of the lactobacilli strains prior to incubation.

The measurement of the coaggregation abilities of the lactobacilli strains with four *C. albicans* strains were determined using a spectrophotometric assay (11). The lactobacilli cell suspensions were prepared similar to the autoaggregation assay. Overnight cultures of *C. albicans* strains in SD broth (Merck, Germany) were washed twice and diluted to 1.0 ± 0.02 at OD_{600} in PBS (pH 7.4). One millilitre of the lactobacilli strains was mixed with equal amount of *C. albicans* strains in sterile test tubes. The mixtures were vortexed for 10 s using a vortex mixer (MixMate, Eppendorf, U.S.A.) before they were incubated aerobically for 4 and 24 h at 37°C. The absorbance of the mixture was recorded at OD_{600} and the percentage of coaggregation between the lactobacilli strains and *C. albicans* were expressed using the formula:

$$\text{Percentages of coaggregation (\%)} = \frac{[(OD_1 + OD_2) - 2\,(OD_3)]}{(OD_1 + OD_2)}$$

Whereby,
OD_1 is the optical density of the lactobacilli strains,
OD_2 is the optical density of *C. albicans* strains,
OD_3 is the optical density of the co-cultured mixture of lactobacilli and *C. albicans*

Microbial adhesion to hydrocarbons (MATH)

The cell surface hydrophobicity of the five selected lactobacilli strains were evaluated using the MATH test formerly described (9) with some modifications. Xylene and toluene (Nacalai Tesque, Japan) were the hydrocarbons utilised in this experiment. The lactobacilli strains were prepared to OD_{600} of 0.5 ± 0.02 in PBS, pH 7.4 as previously described in the aggregation assay. One millilitre of xylene or toluene was added to 3 mL of standardised lactobacilli suspension and incubated at room temperature for 10 min. The mixtures were vortexed for 2 min and incubated for 20 min at room temperature before the upper phase containing the xylene or toluene were removed. The absorbance of the aqueous phase containing the cells was measured at OD_{600}. The percentage of cell surface hydrophobicity was calculated using the formula:

$$\text{Cell surface hydrophobicity (\%)} = 100 \times (1 - OD_1/OD_2)$$

Whereby,
OD_1 is the optical density of the lactobacilli strains after mixing with xylene or toluene,
OD_2 is the optical density of the lactobacilli strains before mixing with xylene or toluene

Acid tolerance assay

The 48 h lactobacilli cultures in MRS broth were rinsed twice with PBS (pH 7.4) before they were measured to 0.5 ± 0.02 at OD_{600} to standardise the lactobacilli cells. The lactobacilli strains were subcultured into 2 mL of MRS broth with varying acidity of pH 1.5, pH 2.5 and pH 3.5 adjusted with 2M of HCl (Merck, Germany) in sterile test tubes. The test tubes containing the cultures were incubated at 37°C, aerobically for up to 24 h. Serial dilutions of the strains were carried out and plated at 2, 4 and 24 h respectively to determine the number of colony-forming unit (CFU/mL) present after exposure to varying levels of acidity.

Bile tolerance assay

The lactobacilli strains were prepared similar to the acid tolerance assay and subcultured into 2 mL of MRS broth containing 0.4%, 0.5% and 0.6% of bile (Sigma Aldrich, U.S.A.), respectively before they were incubated aerobically at 37°C for 24 h. Serial dilutions of the strains were carried out and plated onto MRS agar

at 2, 4 and 24 h, respectively to determine the number of colony-forming unit (CFU/mL) present after exposure to varying concentrations of bile.

Resistance to spermicide

The lactobacilli strains were tested for their ability to resist the effects of spermicides by culturing them on MRS agar with varying concentrations of the spermicide, nonoxynol-9 (N-9) (ab143673, Abcam, U.S.A.) as previously described (12) with slight modifications. The nonoxynol-9 was added at 0.5, 1.0, 2.5, 10.0 %, respectively to molten MRS agar before it was left to solidify. The lactobacilli strains were cultured onto the prepared MRS agar and incubated for 48 h at 37°C under anaerobic conditions. Sterile MRS agar without the presence of nonoxynol-9 were used as blank controls. The plates were examined after incubation to determine the ability of the lactobacilli strains to grow at various concentrations.

RESULTS

Autoaggregation, coaggregation and cell surface hydrophobicity of isolated lactobacilli strains

Aggregation is one of the essential antimicrobial properties needed to prevent the adherence of pathogenic microorganisms. *Lact. reuteri* 29A and *L. delbrueckii* 45E exhibited the highest autoaggregation ability followed by *L. reuteri* 29B, *L. mucosae* 28C and *L. fermentum* 28E (Table I). The strain, *L. reuteri* 29A demonstrated superior ability to aggregate in comparison to the other strains as observed at 4 and 24 h (31.82 ± 5.40 and 79.62 ± 5.92, respectively). Statistical analysis of the readings obtained showed that none of these readings were statistically significant when compared to the commercialised strain, *L. reuteri* RC-14, which was used as the control.

Table I: Percentage of autoaggregation exhibited by lactobacilli strains after 4 h and 24 h incubation

Species	Percentage of autoaggregation (%)	
	4 h	24 h
L. delbrueckii 45E	25.44 ± 13.07	61.27 ± 6.60
L. fermentum 28E	21.11± 7.09	50.47 ± 4.21
L. mucosae 28C	16.28 ± 8.08	51.39 ± 6.57
L. reuteri 29A	31.82 ± 5.40	79.62 ± 5.92
L. reuteri 29B	25.13 ± 7.32	51.92 ± 5.75
L. reuteri RC-14	28.13 ± 6.86	59.75 ± 2.68

Results shown are the means of three independent runs ± SD.

In the present coaggregation assay, *Candida albicans* strains were utilised as they are the main causative agents of vulvovaginal candidiasis (VVC) which is a major fungal infection that affects most women of childbearing age at least once in their lifetime (13). All the lactobacilli strains tested exhibited high coaggregation abilities with the *C. albicans* strains tested as the percentage of coaggregation were between 68 - 92% (Table IIa) at 4 h. *Lact. reuteri* 29A and *L. delbrueckii* 45E exhibited the highest percentage of coaggregation followed by *L. reuteri* 29B, *L. fermentum* 28E and *L. mucosae* 28C (Table IIa and IIb). Statistical analysis of the readings obtained showed that the percentage of coaggregation at 4 h exhibited by *L. mucosae* 28C and *L. fermentum* 28E were significantly ($p < 0.05$) lower when compared to the control strain, *L. reuteri* RC-14. Meanwhile, the statistical analysis of the readings obtained at 24 h demonstrates that *L. mucosae* 28C and *L. fermentum* 28E are significantly lower ($p < 0.05$) and higher ($p < 0.01$) than the positive control, *L. reuteri* RC-14, respectively. The other readings were not significant in comparison to *L. reuteri* RC-14.

The ability of lactobacilli strains to coaggregate is associated with their cell surface hydrophobicity. The cell surface hydrophobicity of the five lactobacilli strains were evaluated by MATH test to measure their absorption to two different hydrocarbons, xylene and toluene (Table III). The results obtained showed that *L. reuteri* 29A (more than 70%) had the highest percentage of absorption to both hydrocarbons followed by *L. reuteri* 29B, *L. mucosae* 28C, *L. reuteri* RC-14, *L. fermentum* 28E and *L. delbrueckii* 45E.

Determination of acid and bile tolerance of lactobacilli as probiotics

Lactobacilli strive in hostile environments due to their ability to tolerate highly acidic environments. The five lactobacilli strains were subjected to three different acidic conditions. *Lact. reuteri* 29B withstood acidic conditions better and longer compared to other lactobacilli strains (Table IV). It survived for up to 4 h at pH 2.5 and 24 h at pH 3.5. The strains, *L. reuteri* 29A and L. delbrueckii 45E could not survive at pH 2.5. They only withstood treatment with low colony-forming units (CFU/mL) for 2 h at pH 3.5.

Lactobacilli should be able to tolerate bile as it is destructive to bacterial integrity. Five of the lactobacilli strains were subjected to three different bile concentrations. As observed with the acid tolerance assay, *L. reuteri* 29B is the only lactobacilli strain that withstood the bile concentrations at 0.4% and 0.5% for up to 4 h. *Lact. reuteri* 29B faced a drastic reduction from the initial, 1 x 10^7 CFU/mL to a mere 50 – 100 CFU/mL after incubation with the bile (Table V).

Resistance to spermicides

Nonoxynol-9 (N-9), the active compound in spermicides is one of the factors that contributes to the changes of the commensal microorganisms (14). The lactobacilli strains were subjected to treatment with four different concentrations of spermicides (0.5%, 1.0%, 2.5% and 10%). *Lact. mucosae* (28C), *L. reuteri* (29B and 29A) and *L. rhamnosus* GR-1 (positive control) were not affected by the presence of spermicide. *Lact. mucosae* (28E) and *L. delbrueckii* (45E) exhibited a drastic reduction in growth at 10% (Fig 1).

Table IIa: Percentage of coaggregation exhibited by lactobacilli strains against several C. albicans strains after 4 h incubation

Pathogen tested	Percentage of coaggregation (%)					
	***L. delbrueckii* 45E**	***L. fermentum* 28E**	***L. mucosae* 28C**	***L. reuteri* 29A**	***L. reuteri* 29B**	***L. reuteri* RC-14**
***C. albicans* ATCC 14053 (Reference)**	86.47 ± 5.68	81.29 ± 10.75	84.66 ± 11.64	92.08 ± 8.01	87.85 ± 8.11	89.96 ± 9.92
***C. albicans* 08**	86.55 ± 5.89	76.05 ± 3.36	75.17 ± 3.95	92.30 ± 8.30	86.85 ± 6.02	84.61 ± 5.35
***C. albicans* 21**	82.78 ± 10.01	76.23 ± 1.23*	77.14 ± 2.78*	91.31 ± 7.36	81.09 ± 5.68	82.56 ± 5.90
***C. albicans* 28**	82.84 ± 9.58	68.79 ± 12.79	77.77 ± 2.46	91.30 ± 7.13	82.06 ± 2.74	75.69 ± 3.23

The results shown are the means of three independent runs ± SD.
* indicates that the values are significant, $p < 0.05$ when compared to the control, *L. reuteri* RC-14.

Table IIb: Percentage of coaggregation exhibited by lactobacilli strains against several C. albicans strains after 24 h incubation

Pathogen tested	Percentage of coaggregation (%)					
	***L. delbrueckii* 45E**	***L. fermentum* 28E**	***L. mucosae* 28C**	***L. reuteri* 29A**	***L. reuteri* 29B**	***L. reuteri* RC-14**
***C. albicans* ATCC 14053 (Reference)**	93.76 ± 1.53	91.68 ± 1.39	89.95 ± 0.89*	95.99 ± 1.55	94.60 ± 2.19	90.94 ± 1.73
***C. albicans* 08**	94.50 ± 0.67	91.94 ± 1.23**	90.89 ± 1.27	96.12 ± 1.33	94.90 ± 2.29	91.90 ± 1.09
***C. albicans* 21**	94.7 ± 1.83	90.29 ± 1.54	91.01 ± 0.94	90.81 ± 8.24	93.19 ± 2.47	91.27 ± 0.60
***C. albicans* 28**	94.31 ± 1.50	90.66 ± 1.79	90.78 ± 0.75	96.29 ± 1.04	94.83 ± 1.03	93.09 ± 1.21

The results shown are the means of three independent runs ± SD.
* and ** indicate that the values are significant, $p < 0.05$ and $p < 0.01$, respectively when compared to the positive control, *L. reuteri* RC-14.

Table III: Cell surface hydrophobicity (%) of lactobacilli strains as determined through MATH test

Species	Cell surface hydrophobicity (%)	
	Toluene	Xylene
L. delbrueckii 45E	12.15 ± 1.66	11.74 ± 5.48
L. fermentum 28E	33.59 ± 9.15	38.27 ± 1.01
L. mucosae 28C	46.82 ± 5.66	54.43 ± 2.85
L. reuteri 29A	73.96 ± 3.09	79.85 ± 10.51
L. reuteri 29B	53.24 ± 10.44	69.81 ± 3.21
L. reuteri RC-14	43.31 ± 3.69	56.56 ± 2.57

Results shown are the means of triplicates ± SD.

Table IV: The colony-forming units (CFU/mL) of lactobacilli after incubation at different acidity for 2, 4 and 24 h

Species	pH 2.5			pH 3.5		
	2 h	4 h	24 h	2 h	4 h	24 h
L. delbrueckii 45E	-	-	-	263	31	-
L. fermentum 28E	-	-	-	66	153	-
L. mucosae 28C	-	-	-	3	7	-
L. reuteri 29A	-	-	-	2.50×10^6	-	-
L. reuteri 29B	3.73×10^5	1.46×10^5	-	2.28×10^6	1.23×10^6	6.90×10^3

The results obtained are the mean of triplicates from three independent. The results for pH 1.5 has been excluded from the table below as there were no growth for any of the lactobacilli strains over the period tested.

Table V: The colony-forming units (CFU/mL) of lactobacilli after incubation with different concentrations of bile for 2 h and 4 h

Species	Colony-forming units (CFU/mL)			
	0.4% bile		0.5% bile	
	2 h	4 h	2 h	4 h
L. delbrueckii 45E	1	-	-	-
L. fermentum 28E	3	-	-	-
L. mucosae 28C	2	-	-	-
L. reuteri 29A	-	-	-	-
L. reuteri 29B	55	130	57	26

The results obtained are the mean of triplicates from three independent experiments. The result for 0.6% bile concentration was excluded from the table below as there were no growth for any of the lactobacilli strains over the period tested.

DISCUSSION

Five of the lactobacilli strains which were isolated from anogenital region of healthy women in our previous study exhibited high antimicrobial activities. They were documented to produce varying amount of lactic acid and hydrogen peroxide that contributed to their antimicrobial activities. In the current study, these lactobacilli strains were further evaluated to determine their suitability as potential probiotics (6).

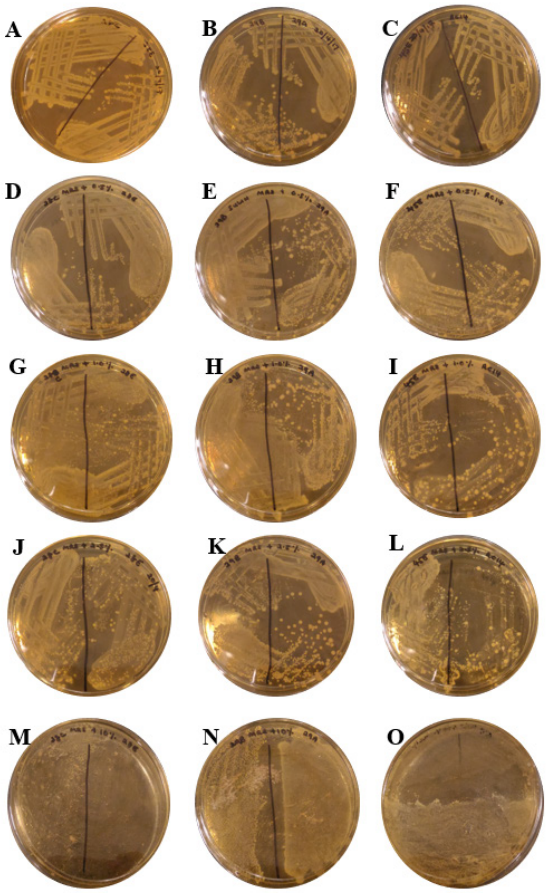

Figure 1: Resistance towards spermicide assay tested on L. mucosae (28C and 28E) (D, G, J and M), L. reuteri (29B and 29A) (E, H, K and N) and L. delbrueckii (45E) (F, I, L and O) with obvious growth on MRS plates containing various concentrations of spermicide (N-9) (0.5%, 1.0%, 2.5% and 10%). L. reuteri RC-14 (positive control) was tested simultaneously with the lactobacilli strains against the same concentration of spermicide. The lactobacilli strains were grown on MRS agar (A, B and C) without the addition of any spermicides (negative control) at the same time.

Aggregation is an important characteristic for lactobacilli to exert their beneficial effects (15). The aggregation characteristics exhibited by lactobacilli are categorised into two groups which are autoaggregation and coaggregation (10). Autoaggregation is a favourable characteristic for probiotics as it would enable them to adhere to host epithelial cells and prevent the colonisation of pathogenic microorganisms (16, 17). In the present study, all the lactobacilli strains exhibited varying degree of autoaggregation abilities.

Lact. reuteri 29A exhibited the strongest degree of autoaggregation phenotype when compared to the other lactobacilli strains followed by *L. delbrueckii* 45E and *L. reuteri* 29B. The percentage of autoaggregation for the control, *L. reuteri* RC-14 fell between *L. delbrueckii* 45E and *L. reuteri* 29B. Autoaggregation among *L. reuteri* was previously noted and some of the cell surface proteins involved have been uncovered (18). *Lact. delbrueckii* previously demonstrated the ability to autoaggregate (19). It is apparent that autoaggregation is not only strain dependent but time dependent as well (10).

Coaggregation enables lactobacilli to form hostile micro-environments containing high concentrations of antimicrobial substances such as lactic acid and H2O2 around pathogens (20). The ability to aggregate with pathogenic microorganisms such as *Candida* species has been described as a desired characteristic for lactobacilli as potential probiotics (15, 20). The five isolated lactobacilli strains exhibited high percentages of coaggregation (between 75 to 90%) against the reference and clinical isolates of *C. albicans*. *Lact. reuteri* 29A exhibited the highest percentage of coaggregation at 4 and 24 h. As observed with the autoaggregation assay, *L. reuteri* 29A, *L. reuteri* 29B and *L. reuteri* RC-14 demonstrated varying coaggregation abilities even though they are of the same species. This result is unsurprising as a similar observation noted that *L. reuteri* DCM 17938 was better at inhibiting the growth of the test *Candida* sp. compared to *L. reuteri* ATCC PTA 5289 (20). The findings indicate that coaggregation is dependent on the variation in strains.

Bacterial adhesion to host epithelial cells is a complex interaction that takes place between bacterial cell membrane and their interacting surface such as receptors (21). One of the most important characteristics that enables bacteria to adhere to host cells is their cell surface hydrophobicity (21). The MATH test has been extensively utilised since its first introduction by Rosenburg et al. (1980) to measure the cell surface hydrophobicity of potential probiotic strains (9, 10, 11). The concept behind this test is to determine whether potential probiotics such as lactobacilli have the physicochemical properties that aids in the colonisation abilities of the microorganisms (22). The lactobacilli strains are often grouped according to their percentage of cell surface hydrophobicity which are either low hydrophobicity or hydrophilic (0 to 35%), moderate hydrophobicity (36 to 70%) and high hydrophobicity (71 to 100%) (10, 23).

Lact. reuteri 29A is highly hydrophobic which tallies with the high readings from the aggregation assays. The percentage of cell surface hydrophobicity for *L. mucosae* 28C, *L. fermentum* 28E and *L. reuteri* 29B seem to reflect their respective percentages in the aggregation assays. However, *L. delbrueckii* 45E which exhibited high percentages of auto- and coaggregation demonstrated the lowest ability to absorb the hydrocarbons tested. Similar results were previously reported (21) whereby, *L. delbrueckii* exhibited the lowest percentage of cell surface hydrophobicity and aggregation capabilities compared to four other lactobacilli strains tested. Low hydrophobicity among several *L. delbrueckii* strains were previously associated with their low exopolysaccaharide (EPS)-producing properties (24).

The general criteria for potential probiotics include evaluating their overall characteristics such as their antimicrobial properties, tolerance, survival and persistence as well as safety (25). The tolerance test would determine the microorganisms' stability in the presence of acid and bile (26) which is necessary for its survival in the hostile host environment.

Tolerance to acidic conditions is a vital characteristic that probiotics should exhibit (27). This property plays an important role in ensuring the survival of probiotics on their journey through the host acidic stomach environment, which normally ranges between pH 2.5 to 3.5 (28). The acidity in the stomach has previously been recorded to drop as low as pH 1.5 during fasting and increase to pH 6.0 or higher after the consumption of food (29, 30). Thus far, the ideal probiotic strains are described to withstand the acidity of up to pH 3.0 (30, 31).

There was no observable growth for any of the lactobacilli strains at pH 1.5 and for most of the lactobacilli strains at pH 2.5, except *L. reuteri* 29B. The ability of *L. reuteri* 29B to survive at pH 2.5 and pH 3.5 for up to 4 h and 24 h, respectively are consistent with a study conducted on *L. reuteri*, whereby more than 80% of the strains tested tolerated the effects of pH 2.7 for an hour (32). The lowered CFU/mL count of *Lact. reuteri* 29B at pH 2.5 and pH 3.5 after 4 h, is parallel with the findings for the commercialised strain, *L. rhamnosus* GG, in which a drastic drop of CFU/mL was noted at pH 2.0 at 4 h (33).

Lact. reuteri 29A and *L. delbrueckii* 45E did not exhibit the same level of tolerance at pH 2.5 and pH 3.5. Moreover, it is observed that the growth of *L. mucosae* 28C and *L. fermentum* 28E were absent at all three acidities tested. The differences in tolerance level between the five lactobacilli strains and those previously reported could be due to species- and strain-dependence. This finding is strengthened by the study formerly conducted (34) which identified one isolate of *L. casei* that was capable of surviving at high acidity among the twenty-eight lactobacilli isolates tested.

Tolerance to bile is considered another important characteristic that probiotics should exhibit. Bile is synthesised from cholesterol in the liver and has demonstrated deleterious effects against bacteria ranging from impairing the bacterial cell integrity to damaging the bacterial DNA (35). It is stored in the gall bladder and released into the duodenum during digestion to aid with the solubilisation and absorption of dietary fats (35). Under normal circumstances, the intestine houses bile salt concentration gradient that ranges between 0.05% and 2% which is approximately about 1 mM to 40 mM (35, 36). Due to its destructive nature towards bacteria, tolerance towards bile by potential probiotics is critical to ensure its safe passage to the colon.

Lact. reuteri 29B demonstrated its tolerance to 0.4% and 0.5% bile at a lowered CFU/mL. The same cannot be said for *L. reuteri* 29A which showed a lack of growth at all concentrations. The current observation for *L. reuteri* 29A and 29B differed from the findings reported (37) whereby, *L. reuteri* strains were able to survive treatment with 0.5% porcine bile for 72 h, with only a slight reduction in CFU/mL. Although our findings are reflective of the acid tolerance assay in which, *L. reuteri* 29B is the most tolerant strain among the five strains. The increased tolerance towards bile exhibited by *L. reuteri* has been associated with the presence of multidrug transporters (MDRs) which has been said to reduce toxicity by pumping out the excess bile and salt (35). It is observed that the lack of growth for the other lactobacilli strains at high concentrations of bile were mirrored in their inability to survive at low pH thus, indicating that there might be an association between the mechanisms required for their survival at low pH and high bile concentrations.

Lact. mucosae 28C, *L. fermentum* 28E and *L. delbrueckii* 45E showed a low CFU/mL at 0.4% for 2 h. The outcome noted for *L. mucosae* 28C cannot be compared with previous study as there are not many data available on its ability to tolerate bile. The results obtained for *L. fermentum* 28E differed from a previous report which showed that *L. fermentum* can withstand the treatment with 0.5% bovine bile for up to 72 h (37). However, thirty-seven *L. fermentum* strains were previously identified for their inability to adapt to 0.3% bile thereby, indicating that our findings are not uncommon (38). Our finding is in contrast with that formerly reported (38) in which, *L. delbrueckii* strains were able to tolerate various bile concentrations. It is however, consistent with the reports of a former study (39) which observed that several strains of *L. delbrueckii* used in their study could not tolerate treatment with bile salt. As observed with the previous aggregation and acid tolerance assay, strain- and species-dependence seems to play a role in lactobacilli tolerance of bile.

Spermicide is a common contraceptive utilised by women as a form of birth control. The active compound in most of these spermicides, nonoxynol-9 (N-9) is found at 5% concentration in commercialised spermicides. It is a nonionic detergent that has also been described as having inhibitory effects against the vaginal microbiota such as lactobacilli (40). N-9 demonstrated the ability to incapacitate the antimicrobial activities of commensal lactobacilli thereby, enabling E. coli to thrive (41). Over time, there have been increasing reports regarding the risk of infections due to the disruption of the vaginal flora (42). The increase in infections has been reported to be due to the disruptive nature that these types of contraceptives induce towards the normal vaginal flora (38). As spermicides are quite commonly utilised, it is crucial to find probiotic strains that are able to withstand

high concentrations of N-9.

Lact. reuteri 29A and *L. reuteri* 29B were able to grow at all concentrations of N-9 tested. There was a slight reduction in growth for *L. delbrueckii* 45E, *L. fermentum* 28E and *L. mucosae* 28C at a higher concentration of N-9 (10%). According to Paauw (2013), N-9 is toxic to hydrogen-peroxide (H2O2) producing lactobacilli (43). The decrease in growth of *L. delbrueckii* 45E, *L. fermentum* 28E and *L. mucosae* 28C, the highest producers of H_2O_2 (reported in our previous paper) could be associated with that. Similarly, the weakest H_2O_2 producers, *L. reuteri* 29A and *L. reuteri* 29B appeared unaffected by N-9 concentrations. A comparison of *L. reuteri* 29A and *L. reuteri* 29B against commercialised strain, *L. reuteri* RC-14, once again highlights the role of strain and species differences in probiotics.

CONCLUSION

Out of the five lactobacilli strains that demonstrated high antimicrobial properties, *L. reuteri* 29B exhibited the most probiotic traits with high degree of auto- and co-aggregation and high tolerance against acid, bile and spermicide. Our findings do not imply that the remaining lactobacilli strains are inapt probiotics however, these strains are probably unsuitable for direct oral administration. The present study once again indicates that the anogenital region is a potential anatomical site to search for new probiotic strains.

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