Study on the functional properties of indigenous probiotics isolated from human samples in West Africa

Abstract

A study was performed to identify and characterize 99 indigenous strains isolated from human breast milk (n=29) and fecal samples (n=70) in Ghana, using methods ranging from plating growth tests, conforming to (NCCLS) standards and presumptive analysis at species level using MALDI-TOF MS, prior to validation through qPCR techniques. Antimicrobial activities of the isolated strains were performed by the agar well diffusion assay. Gastrointestinal tolerance and ability to grow biofilms *in vitro* using 3D Alvatex platform were determined. Results indicate that out of 99 samples, 25 were gram-positive, catalase-negative rods, 80% of which were *Lactobacillus* strains. More than one-third of the identified strains were *L. fermentum* followed by *L. plantarum, L. rhamnosus, L. salivarius, L. reuteri* along with *Enterococcus faecium*, *Weissella* spp. and *Pediococcus* spp. Among the 25 isolates, 9 had activity against both gram-negative and gram-positive tested pathogens including *Staphylococcus aureus*, *Escherichia coli* ATCC 25922, *E. coli* BAA-2471, *Salmonella typhi,* and *Pseudomonas aeruginosa.* The indigenous microbiota proved to be most effective antimicrobial to local pathogens over nonindigenous pathogens. The data also showed maintained cell viability for all studied isolate at pH 2, 3 and 6 followed by a successful growth of the co-cultured biofilm in a 3D Alvatex platform.

Keywords: Microbiome; Probiotics; Lactic Acid Bacteria (LAB); Lactobacillus; indigenous strains

1 Introduction

During the 20th century, little was known about the beneficial attributes of some commensal bacteria before the noble laureate Elie Metchninkoff introduces the concept of probiotics and their boosting effects on human health (Metchnikoff, 1908). In view of this original contribution, various studies have then been implemented by microbiome researchers including the World Health Organization (WHO) to ascertain the health benefits from an adequate amount of probiotics to the host. Among commensal microorganisms pertaining to fermented food and dairy industry (i.e., Yogurt, Kefir and cheese), lactic acid bacteria (LAB), primarily species descending from the *Lactobacillus* genera, have been extensively used for their fermentative ability and probiotic potential (FAO/WHO, 2001; Linares, Ross, & Stanton, 2016). Within LABs, some *Lactobacillus* strains constitute the most prevalent components of the gastrointestinal tract (gut) and vaginal microbiota (Metchnikoff, 1908; Westerik, Kort, Sybesma, & Reid, 2018). These strains can form biofilms in the host cells and protect them from infective bacteria such as mastitis pathogens, by producing bacteriocins, which act as antimicrobials, in addition to hydrogen peroxide and lactic acid (Ocana & Nader-Macias, 2004). The most common species of *Lactobacillus* strains used in food have been extensively documented (Salas-Jara, Ilabaca, Vega, & Garcia, 2016; Tamang, Watanabe, & Holzapfel, 2016). Furthermore, *L. rhamnosus* GG ATCC 53103 (Valio) along with *L. rhamnosus* GR-1 as well as *L. paracasei* Shirota (Yakult) and *B. lactis* BB12 (Chr. Hansen) are the world's most documented probiotics, which also dominate the probiotic market. These species originating from America, Asia and Europe have shown high efficacy in the management of lactose intolerance, immune response modulation, and protection against *Clostridium difficile* and *Helicobacter pylori* infections. According to Linares et al. (2016) and Westerik et al. (2018), they also protect bacteria and fungi in the urogenital, gut, and respiratory tract, as well as against rotaviral and antibiotic-associated infections along with Travelers' diarrhea.

While controlled trials have shown that some strains are able to reduce the symptoms of irritable bowel syndrome (IBS) and inflammation in ulcerative colitis (Guslandi, 2007) and the rate of rotavirus-associated diarrhea (Dubey, Rajeshwari, Chakravarty, & Famularo, 2008), other studies suggest that these probiotics have potential to increase cure rate for bacterial vaginosis (Martinez et al., 2009) and decrease incidence of allergic diseases (Abrahamsson et al., 2007) as well as, postoperative complications of colorectal cancer (Zhang et al., 2012). They also have been shown to improve quality of life in children with cystic fibrosis (Jafari et al., 2013). Furthermore, recent research has suggested in addition to the functional properties of the human microbiota, the latter also plays a role in brain development and can be linked to autism, depression, anxiety and stress (Abildgaard, Elfving, Hokland, Wegener, & Lund, 2017; Kraus, Cetin, & Aricioglu, 2016; Nduti et al., 2016; Sawada et al., 2017; Severance et al., 2017).

Although these species have been extensively applied in Africa (Bisanz et al., 2014; Nduti et al., 2016), there is an unmet need to isolate indigenous strains, which have adapted to local conditions and have potential to be more specific to the local population. Various probiotics and their potential benefits have been identified and reported, however, there is limited information on isolated and documented indigenous probiotic microorganisms. While there is a research study on potential probiotic isolates from fermented food in Ghana (Owusu-Kwarteng, Tano-Debrah, Akabanda, & Jespersen, 2015), little is known about their benefits to the residents. We believe that the indigenous flora has a high potential to be valuable in the management of enteric infections, boost immunity, and generally improve the wellbeing of local population that are often exposed to poor hygiene condition, malnutrition, and chronic enteric infections. Moreover, with our understanding of how an individual's genetics, environment, and diet influence their microbiota and the current trend towards personalized medicine (Vogenberg, Isaacson Barash, & Pursel, 2010), it has become very important to identify local strains, those which predominate in the specific population. This avoids the one-size-

fits-all approach and generalization of the effectiveness of probiotic species, which often results in lack of efficacy (Allen et al., 2013; Ritchie & Romanuk, 2012). Locally sourced probiotics are of prime interest pertaining to their immediate availability in the local market and high antimicrobial specificity to residents' host cells.

The objective of this study is to identify and characterize the functional properties of locally isolated probiotics from human feces and breast milk samples in Ghana to ascertain the beneficial effects of indigenous probiotics over the non-indigenous strains. Particular focus of this study is to elucidate the prevalence of the most significant probiotics including *Lactobacillus* strains and determine their antimicrobial activities, biofilm growth ability and resistance to acidic environment. The standards and presumptive identifications were performed using MALDI- TOF MS followed by qPCR techniques. While the antimicrobial activity was tested by the agar well diffusion assay, the *in vitro* growth of biofilm and the gastrointestinal tolerance were demonstrated using 3D Alvatex platform.

2 Materials and methods

2.1 Isolation and phenotypic identification of LAB species

Fecal samples were obtained from five adults, one adolescent, three children, and three infants. Milk was obtained from six breastfeeding mothers. 1 g of fecal sample was homogenized in 10 mL of phosphate buffered saline (PBS, pH 7.4) using a vortex. A loopful of the fecal suspension and breast milk sample was streaked onto separate de Man Rogosa Sharpe (MRS) broth (Oxoid) supplemented with 0.05% w/v L-cysteine hydrochloride and 0.002% w/v of bromophenol blue, and incubated anaerobically at 37ºC for 48 h. Colonies with different morphological characteristics were picked from the agar plates and cultured on MRS agar supplemented with only 0.05% L-cysteine hydrochloride (MRSc). The plates were incubated at 37ºC for 48 h anaerobically. Pure colonies obtained were Gram-stained and observed under the microscope. Biochemical profile such as the

catalase test was performed on the isolates. Out of 99 colonies isolated including 29 colonies from breast milk samples and 70 from fecal samples, 25 of the isolates were gram-positive rods and 80% of the isolated species were identified by MALDI-TOF MS or 16S rRNA gene sequencing as belonging to *Lactobacillus* genus.

2.2 Identification of isolates using MALDI-TOF

Isolates were identified by Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) using the MALDI Biotyper®. Briefly, a single colony of freshly grown isolated bacteria was smeared as a film onto individual spots on MALDI-target plate using 1 µL disposable loop and allowed to air dry. The film was overlaid with 1 µL of a 98% formic acid solution to allow on-plate extraction of cellular proteins. After drying, the film was further overlaid with 1 µL of a MALDI-TOF MS matrix comprising of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 2.5% trifluoroacetic acid. The sample was further air-dried for 1-2 min and analysed with the Bruker Microflex LT bench top MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA), Bruker FlexControl 3.0 software, and MALDI Biotyper 3.1 software (Bruker Daltonics, Billerica, MA, USA). The reference strain was *Escherichia coli* ATCC 8739. Mass spectra for each spotted bacterial isolate was acquired with the instrument in a linear positive mode within a 2-20 kDa range, with ion source 1.0 at 20 kV, ion source 2.0 at 18.05 kV, the lens at 6.0 kV, and the linear detector at 2,560 V. Mass spectra was analyzed and compared with the MALDI Biotyper 3.1 software database, comprised of 4,970 distinct bacterial species, to determine the most likely microbial genus and species identification. A MALDI Biotyper score, generated as a level of probability by the software, of ≥ 1.7 was utilized as a threshold for reliable species identification, as recommended for assessment of anaerobic bacteria (Hsu & Burnham, 2014). The

MALDI-TOF MS has previously been used for bacterial identification and established as an alternative to bacterial identification providing rapid determination (Levesque et al., 2015; McElvania TeKippe & Burnham, 2014).

2.3 Identification of isolates using Polymerase Chain Reaction (PCR)

The genomic DNA of the isolated bacteria was extracted using the GenElute™ Bacterial Genomic DNA Kit (Sigma) according to the manufacturer's protocol. The 16S rRNA gene was amplified using the following primers: 8F, 50-AGAGTTTGATCCTGGCTCAG-30, and 1492R, 50-GGTTACCTTGTTACGACTT-30. PCR was performed using the MJ Mini™ Thermal Cycler (Bio-Rad). The reaction contained in 50 µL total volume, 0.5 µM of each primer (0.5 µL of each), 1 µL of genomic DNA, 25 µL GoTaq® Master mix (Promega; reaction buffer, 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$) and 23 µL RNase free water. The PCR was run under an initial activation at 94ºC for 2 min, followed by 30 cycles of denaturation step cycles at 94ºC for 30 s, an annealing step at 55ºC for 1 min, extension step at 72ºC for 1 min and final cycle at 72ºC for 10 min. 9 µL aliquots of the PCR samples were subjected to electrophoresis in 0.8% w/v agarose gel (stained with 0.5 µg/mL ethidium bromide), in TAE buffer. The gel was visualized under UV illumination and photographed. The PCR samples were further purified and sequenced.

2.4 Antimicrobial activity of isolates

Antimicrobial activity of the isolated species was tested by the agar well diffusion assay against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* BAA-2471 (multi-drug resistant strain), *Salmonella typhi* (local clinical isolate), *Pseudomonas aeruginosa* (local clinical

isolate) and *P. aeruginosa* ATCC 10145. Briefly, 24 h culture of the isolated species in MRSc were centrifuged at 3500 *g* at 4ºC. The supernatant was collected and filter sterilized using a 0.2 µm membrane syringe filter. A lawn of each test-isolated microorganism was made by picking bacterial colonies and spreading on Mannitol Salt agar (Oxoid), MacConkey agar (Oxoid), Cetrimide agar (Oxoid), Bismuth sulphite agar (Oxoid) or Tryptic Soy agar (Sigma-Aldrich). Wells of 9 mm in diameter were made in the agar and filled with 150 µL of the filter-sterilized supernatant. The plates were kept on the bench for 2 h for diffusion of the supernatant and then incubated at 37ºC. Zones of inhibition were recorded after 48 h of incubation.

2.5 Gastrointestinal tolerance assay

Simulated gastric and intestinal juice

Tolerance of selected isolated species to simulated gastric fluid and bile salt were performed by inoculating 100 µL of a culture of each species in 1 mL solution pH adjusted (HCl /NaOH) to 1, 2, 3 and 6 and 0.3% w/v bile salt (Sigma-Aldrich). The cells were incubated at 37ºC and sample taken at 1 h, 2 h and 3 h, serially diluted in PBS (pH 7.4) and spread-plated on MRSc. Samples for bile salt test were further sampled at 4 h. Colonies were counted after incubation at 37ºC for 48 h.

2.6 Biofilm assay

The ability of selected probiotic species to form a successful biofilm growth *in vitro* was evaluated. The Alvatex strata 3D scaffold inserts (ReproCELL Europe Ltd., Glasgow, United Kingdom) were used for simulating the structural matrix found in the gut and mimicking *in vivo* biofilm growth of the bacteria. Each species was grown separately in MRSc broth as previously described. The 3D

scaffold inserts were placed carefully into wells of the plate. The scaffolds were washed with 400 µL of 200-proof ethanol, then 550 µL of sterile PBS. 550 µL of sterile PBS was again poured carefully into the wells making sure the 3D fibrous scaffolds lay flat without any folding. The 3D fibrous scaffolds were UV sterilized for 45 min. The PBS was removed carefully from the wells and replaced with 550 µL of sterile MRSc, which was also carefully removed. 400 µL bacterial culture were inoculated into the wells containing the UV sterilized 3D fibrous scaffold and incubated at 37ºC for 5 days with addition of MRSc as necessary. At the end of incubation the biofilms were stained with the FilmTracer™ LIVE/DEAD™ (Invitrogen™) Biofilm Viability kit (a two-colour fluorescence assay of bacterial viability: SYTO® 9 green fluorescent nucleic acid stain and red-fluorescent nucleic acid stain, propidium iodide) and imaged using EVOS® FL Cell Imaging System at 40X resolution.

3 Results

3.1 Isolation and identification of isolates

Several isolates were obtained from fecal (adults, adolescent, children and infants) and breast milk samples on MRSc media. The isolates were Gram-stained, analyzed by microscopic observation and catalase test. Out of 99 colonies isolated including 29 colonies from breast milk samples and 70 from fecal samples, 25 of the isolates were gram-positive rods and catalase negative. The 25 gram-positive catalase negative rods were submitted to MALDI-TOF MS and 16S rRNA gene sequencing analysis (Table 1). More than 80% of the isolated species were identified by MALDI-TOF MS or 16S rRNA gene sequencing as belonging to *Lactobacillus* genus. More than one-third of the species of were identified as *L. fermentum* by both identification methods. Other species isolated included *L. plantarum*, *L. rhamnosus*, *L. salivarius*, *L. reuteri*, *Enterococcus faecium*, *Weissella* spp. *and Pediococcus* spp. The Jaccard's similarity coefficient between the MALDI-TOF MS and 16S rRNA gene sequencing methods was almost 70%. Isolate 8 and 9 were identified by both methods as

Weissella and *Pediococcus* species respectively. However the *Weissella* species was identified as *Weissella confusa* with MALDI-TOF MS and *Weissella cibaria* with 16S rRNA gene sequencing whilst the *Pediococcus* species was identified as *Pediococcus pentosaceus* and *Pediococcus acidilactici* with both methods respectively. Another isolate identified as *L. reuteri* by MALDI-TOF MS was identified as *L. fermentum* by 16S rRNA gene sequencing whilst a *L. plantarum* was identified as *L. rhamnosus*.

3.2 Antimicrobial activity of isolates

The antimicrobial activities of isolated species were tested against gram-negative *P. aeruginosa* (ATCC 101145 and local clinical isolate), *E. coli* (ATCC 25922 and BAA-2471 multi-drug resistant), *Salmonella typhi* (local clinical isolate) and gram-positive *S. aureus* (ATCC 29213). Only 9 out of 25 isolates had activity against both gram-negative and gram-positive tested bacteria, Table 2. Isolates FSD1-D and FSI3-L showed highest activity against both the gram-negative and gram-positive bacteria, followed by FSD4-D, FSC3-L and FSD3-WC. All tested isolate had activity against *P. aeruginosa* and *E. coli*. Only one isolate, FSD4-I did not show activity against *Salmonella typhi*. Also 13 isolates (almost 60%) did not show zone of inhibition towards *S. aureus*. Isolate FSI3-L had highest activity against *S. aureus* whereas FSD1-D had highest activity against *Salmonella typhi.* The relative activity of the isolates against *P. aeruginosa* was greater in the local isolate than the reference strain. The zone of inhibition against the multi-drug resistant *E. coli* strain was correspondingly smaller than in the reference strain.

3.3 Gastrointestinal tolerance assay

The viable counts of selected isolates to pH 1, 2, 3, 6 and 0.3% bile salt concentration are shown in Tables 3 and 4. The data shows maintained cell viability for all studied isolate at pH 2, 3 and 6. However, all isolates lost total viability during the acid test at pH 1. The exposure of the isolates to 0.3% bile salt concentration showed general maintenance of viability over the 4 h exposure period, Table 4.

3.4 Biofilm assay

The ability of selected isolates to form biofilm was evaluated using the Alvatex 3D scaffold insert (Fig. 1). The confocal imaging (Fig. 1b) of the 3D scaffold at 60x magnification shows randomly aligned fibers associating to form a fibrous mesh. The 3D scaffold was explored to simulate the structural matrix found in the gut and mimic the *in vivo* biofilm growth of bacteria. All tested species formed biofilm on the 3D scaffold (Fig. 2). The biofilm formed for all species comprised of both live and dead cells. All scaffolds maintained integrity during the period of assay except for scaffold used for culturing isolate FSD1-D.

4 Discussion

In the present investigation, we identified twenty-four potential indigenous probiotic isolates as *L. fermentum* (14), *L. plantarum* (2), *L. rhamnosus* (1), *L. salivarius* (1), *L. reuteri* (1), *Enterococcus faecium* (2), *Weissella* spp. (2) and *Pediococcus* spp. (1) from breast milk and fecal samples using the MALDI-TOF MS and 16S rRNA gene sequencing analysis. The predominant species in both the breast milk and fecal samples was *L. fermentum*. Other studies have reported a similar profile for fecal samples (Mandal, Jariwala, & Bagchi, 2016), more diverse profile for breast milk samples

(Damaceno et al., 2017; Khalkhali & Mojgani, 2017) or only one type of bacteria from breast milk (Martin et al., 2003). *Weisella confusa* and *Weisella cibaria* very closely related species with very high 16S rDNA sequence similarity could not be differentiated by one of the two methods in the present study and previously misidentified (Bjorkroth et al., 2002). *Pediococcus pentosaceus* and *Pediococcus acidilactici* are also highly phylogenetically related; 16S rRNA sequence homology of 98.3% (Collins, Williams, & Wallbanks, 1990; Mora, Fortina, Parini, & Manachini, 1997) could not be differentiated by one of the methods. Still, isolate FSI1-D was identified as *L. reuteri* by MALDI-TOF MS and as *L. fermentum* by 16S rRNA gene sequencing analysis (97.75% identity score). It must be noted that *L. fermentum* and *L. reuteri* were previously classified as a single species, because *L. fermentum* is closely related phenotypically but have subsequently been separated (Klein, Pack, Bonaparte, & Reuter, 1998). It is interesting that in the present study, one of the two methods could not differentiate the species. The data also showed a Jaccard's similarity coefficient of 84.62% for the identification of *L. fermentum* and an overall similarity coefficient of approximately 70% for the identification of LAB. 16S rRNA gene sequencing analysis is often considered the gold standard for identification of bacteria and it is commonly combined with phenotypic methods/biochemical fermentation strips for identification of LAB, with the latter methods, streamlining the number of species to be identified; however, for routine use in laboratories, 16S rRNA gene sequencing analysis is limited by its time consuming nature and cost. MALDI-TOF MS offers the advantage of a rapid turn-around time and minimal cost and has been previously demonstrated to be highly congruent (86.1%) to 16S rRNA gene sequencing analysis (Garcia et al., 2016). The present data suggests a good congruency between the two methods at the genus level. However it also brings to light the fact that either single identification method may not be sufficient to identify accurately at the species level. 16S rRNA sequencing has been previously suggested to provide more than 90% genus identification and 65%-83% in species identification (Janda & Abbott, 2007; Mignard & Flandrois, 2006).

249 It is important that potential probiotic candidates produce extracellular antimicrobial compounds to
250 kill pathogenic bacteria and mitigate infectious diseases. The extracellular antimicrobial compounds
251 may include organic acids, hydrogen peroxide, bacteriocins, low-molecular mass peptides and
252 enzymes. When evaluated for the antimicrobial activity against both gram-negative and gram-
253 positive bacteria, with the exception of FSD4-I, which did not show activity against *Salmonella*
254 *typhi*, all the isolates demonstrated inhibitory activity against the tested gram-negative bacteria. The
255 several isolated species of *L. fermentum* exhibited different inhibitory profile against the tested
256 bacteria suggesting strain specificity of activity also observed by Arena et al. (2016) when studying
257 the antimicrobial activity of several isolated *L. plantarum* strains against *Listeria monocytogenes*,
258 *Salmonella Enteritidis*, *E. coli* O157:H7 and *S. aureus.* McCoy and Gilliland (2007) also
259 demonstrated variations of some isolated *L. reuteri* species in inhibiting growth of *Salmonella*
260 *Typhimurium* noting the ability of some *L. reuteri* species to produce reuterin whilst others did not. In
261 the present study, it was noted that the isolates were generally more effective against the local clinical
262 isolate of *P. aeruginosa* than *P. aeruginosa* ATCC 10145. This observation supports that indigenous
263 isolates may be more effective in the treatment of native infections and also more specific to the
264 microbiota of the local population. However the reason for this is not currently known but may be
265 explained to be possibly the previous evolvement and association of the potential probiotic isolates
266 (from the microbiota) with the local *P. aeruginosa.* The species were also more effective against the
267 gram-negative species than the gram-positive *S. aureus*. However, this greater effectiveness towards
268 gram-negative bacteria was not reported by Mandal et al. (2016) when they assessed isolated LAB
269 against *E. coli*, *P. aeruginosa*, *Salmonella Typhi*, *Shigella dysenteriae*, *Proteus vulagaris* and *S.*
270 *aureus* in a similar study. Tharmaraj and Shah (2009) reported a higher inhibition of gram-positive
271 bacteria including *S. aureus* than gram-negative bacteria when evaluating the antimicrobial effects of
272 probiotics against selected pathogenic and spoilage bacteria in cheese-based dips (Tharmaraj & Shah,
273 2009). On the other hand, Tirloni et al. (2014) reported a higher susceptibility of gram-negative

bacteria to antimicrobial activities of *L. animalis* and *L. paracasei* although *P. aeruginosa* was one of the least susceptible species (Tirloni et al., 2014) .

For probiotic species to exert their activity, they must first colonize the gastrointestinal tract. The ability of selected isolates to survive gastrointestinal tolerance was tested in pH 1 to 6 and 0.3% w/v bile salt concentration for 3 h and more. None of the tested isolates survived at pH 1 for the period tested and for 30 min after inoculation (results not shown). All tested isolates were resistant to pH 2 and above and the bile salt. The human stomach has pH ranging from 1 to 2.5 in the fasted states but can peak to 6.7 in the fed state (Dressman et al., 1990; Evans et al., 1988). Although most of the harsh conditions of the gastric environment is contributed by the pH, other enzymatic and digestive substance contained in the gastric fluid as well as the presence of food and the delivery matrix for the probiotic bacteria could affect the survival of the cells during gastrointestinal transit (Fredua-Agyeman & Gaisford, 2015). The results indicate that the tested isolates may survive passage through the gastrointestinal at those pH and bile salt concentration; however, a more biorelevant test may be required. The resistance of the isolates was better than previously reported (Garcia et al., 2016; Jacobsen et al., 1999).

The establishment of the biofilm forming potential of the isolates is very important as the bacterial association within the gut predominantly exist as biofilms. Biofilm provide colonization resistance; they prolong the residence of ingested bacteria, suppress pathogenic microorganisms and interact with the host cells to regulate immunity (de Vos, 2015; Sassone-Corsi & Raffatellu, 2015). The examined species in the present study all showed biofilm forming potential on 3D nanofibrous scaffold. It was however observed that some of the species were self-inhibitory, having a greater proportion of dead than live cells at the end of incubation period. Although quantification of metabolic products such as organic acids, hydrogen peroxide was not made in the present study, the pH measurements of supernatants produced by the species suggest organic acids may have played a

298 role. The lowest pH (pH of 3.8) was recorded for FSD1-D, which digested the 3D scaffold (Fig. 3)
299 followed by FSC3-LBC (pH 4.2). The digestion of the scaffold however could not be replicated in
300 HCl at similar pH.

301 Ultimately, the indigenous microbiota proved to be the most effective antimicrobial for residents over
302 the nonindigenous probiotics, in addition to their potential to regulate host serotonin biosynthesis in
303 the gut as was previously documented by Yano et al. (2015). Our research study herein, demonstrated
304 the capability of some indigenous strains of *Lactobacillus* to effectively inhibit pathogens in the gut
305 and withstand severe *in vivo* conditions, suggesting their combination with commercialized
306 probiotics in order to optimize the indigenous efficiency.

307 They also have exceptional ability to withstand extreme pH conditions. Although our preliminary
308 data on the antibiotic resistance profiling of some strains (results not shown) revealed the presence of
309 some probiotics carrying resistance, future research studies and directions will be focused towards
310 further investigation on the antibiotic resistance profiling to ensure the selection of strains that are
311 free from resistance, which will warrant the benefits and safe-use of this natural therapy. Perhaps, the
312 biofilm will also be enhanced towards the fourth-generation encapsulation to ensure greater *in vivo*
313 bioavailability and potency towards pathogens in the gut.

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7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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List of figures

List of Tables