

Screening and molecular identification of lactic acid bacteria from *gari* and *fufu* and *gari* effluents

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Abstract Bacterial strains were isolated from cassava-derived food products and, for the first time, from cassava by-products, with a focus on *gari*, a flour-like product, and the effluents from the production processes for *gari* and *fufu* (a dough also made from cassava flour). A total of 47 strains were isolated, all of which were tested to determine their resistance to acidic pH and to bile salt environments. Four of the 47 isolates tested positive in both environments, and these four isolates also showed antibacterial behaviour towards both Gram-positive and Gram-negative microbial pathogens (i.e. Methicillin-resistance *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella enteritidis*, *Escherichia coli*, *Escherichia coli* (O157), *Yersinia enterocolitica*). In most cases, the antibacterial activity was related to bacteriocin production. Molecular identification analysis (16S rDNA and randomly amplified polymorphic DNA-PCR) revealed that the four isolates were different strains of the same species, *Lactobacillus fermentum*. These results demonstrate that bacteria isolated from cassava-derived food items and cassava by-products have interesting properties and could potentially be used as probiotics.

Keywords Cassava · Lactic acid bacteria · *Lactobacillus fermentum* · Antibacterial · Waste valorization

Introduction

The increasing awareness of consumers towards a healthy lifestyle has resulted in an ever-growing demand for food products with versatile health benefits including, for example, food items containing probiotic bacteria. The term probiotics refers to live microbial cultures which, when consumed by people or animals (in the form of dehydrated cells or fermented products), can positively affect their health by improving the properties of the original microbiota (Sathyabama et al. 2014).

Due to the prolonged use of antibiotics as infection treatments, more and more pathogenic bacteria have developed resistance to these molecules (Galán et al. 2013). There is, therefore, a need for microorganisms which are not hazardous to human health and which at the same time are effective against such pathogens. One possible mechanism is when healthy bacteria produce substances which are harmful to the pathogens or when they can compete with them for space and nutrients (i.e. colonizing the intestinal cells of the colon).

The isolation and screening of microorganisms from natural sources has always been a very powerful strategy to obtain useful and genetically stable bacterial strains (Adnan and Tan 2006). For example, it is well known that spontaneously fermented foods with mixed cultures are potential substrates to grow bacterial strains. Indeed, in many cases such microorganisms show stable properties, and they are particularly able to withstand stress factors due to the complex environment they were isolated from. Lactic acid bacteria (LAB) are a very important microorganism group comprising several probiotic bacteria, among which *Lactobacillus* sp. has been reported to be the most active and safe (i.e. non-pathogenic)

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microorganism (Salminen and Von Wright 1998). The symbiotic effect of these strains with *Bifidobacterium* sp., another probiotic strain, has also been reported (Kailasapathy and Chin 2000).

Probiotic bacteria have been extensively studied, and this has led to the development of a variety of probiotic foods, especially those involving dairy milk (Ukeyima et al. 2010). While it is fermented dairy foods which have been conventionally associated with probiotics, cereal-based products have also been developed, mainly through the combined use of probiotics, prebiotics and dietary fibres (Lamsal and Faubion 2009; Sanni et al. 2013).

For probiotic bacteria to be effective they must possess a number of specific properties. One such property is the ability to survive in acidic and bile-containing media as they have to undergo these conditions during their passage through the gastrointestinal tract (Klaenhamer and Kullen 1999). Moreover, to have beneficial effect on human health, among other eventual functional properties they must show antibacterial behaviour towards pathogenic strains, either by producing antimicrobial agents (bacteriocins, organic acids, etc) or by reducing the adhesion of pathogenic bacteria (Gareau et al. 2010).

Cassava (*Manihot esculenta*, Crantz) is a very important crop in many African, Asian and South American countries, and according to Food and Agriculture Organization data, it is the fourth most consumed crop worldwide (Ferraro 2016). Nigeria is the primary world producer of cassava, with a production of 37.5 million tons reported in 2010 (Ishola et al. 2013). Indeed, many traditional Nigerian dishes are based on and/or derived from cassava, generating wastes in various forms, both solid and liquid.

As reported in literature, several cassava-derived food products and by-products can be good sources of LAB (Anyogu et al. 2014; Avancini et al. 2007; Banwo et al. 2012; Ouoba et al. 2010; Wilfrid Padonou et al. 2009). *Gari* and *fufu* are two traditional cassava-based Nigerian dishes, both obtained through fermentation processes (Anyogu et al. 2014); therefore, both products and/or by-products can be used for LAB isolation.

In the study reported here, LABs were isolated from cassava products and/or wastewaters from the food production processes, specifically, the effluents from *gari* and *fufu* production as well as *gari* itself. The isolates were characterized to assess their potential activity as probiotic bacteria, and their acid and bile resistance were tested, together with their antibacterial activity towards several pathogenic strains. Species identification and subtyping of isolates were performed by 16S rDNA gene sequencing and M13 randomly amplified polymorphic DNA (RAPD)-PCR typing due to the rapidity and reliability of these techniques in

differentiating LAB strains (Baruzzi et al. 2000; Rossetti and Giraffa 2005).

Materials and methods

Sample collection and storage

Samples used for bacteria isolation were obtained from *fufu* and *gari* processing sites at Abeokuta, Nigeria, stored at 4 °C and used within 48 h. A flow chart of the *fufu* and *gari* production processes is shown in Fig. 1a, b, respectively. Bacterial strains

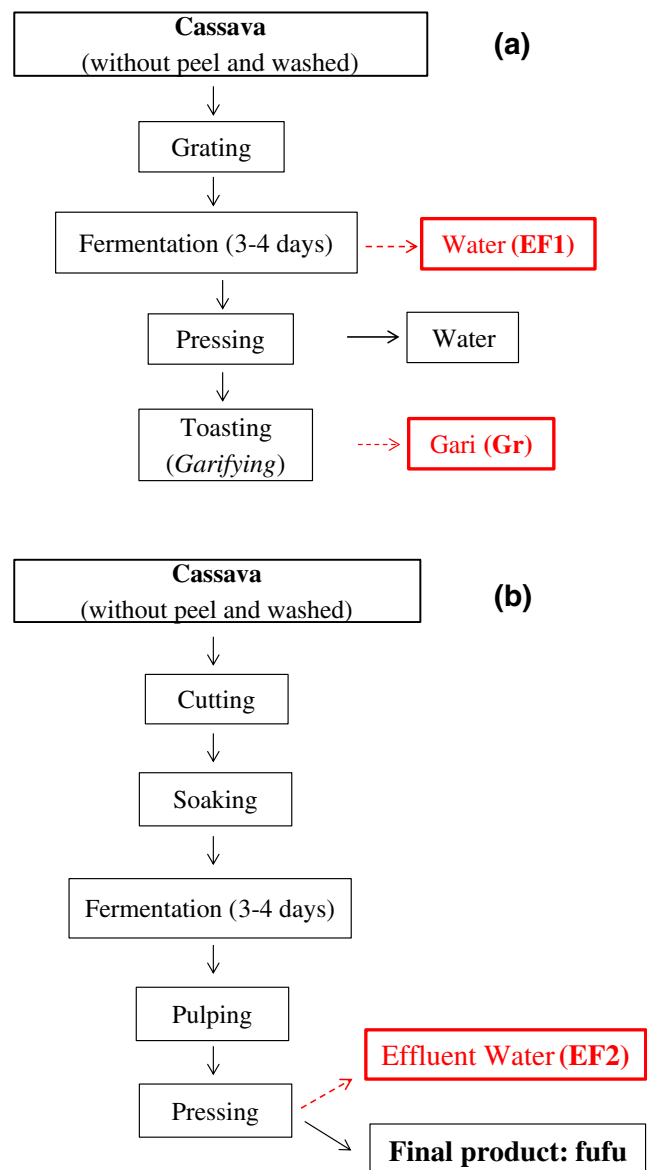


Fig. 1 Flow sheet of *gari* (a) and *fufu* (b) production processes and samples used for analysis. Bacteria were isolated from the effluents of the *gari* (EF1) and *fufu* (EF2) production processes and from the production of the *gari* food item itself (Gr)

were isolated from the effluents of gari (EF1) and fufu (EF2) and from gari itself.

Isolation of LAB

As a first screening, with the aim to select the most suitable medium for isolating LAB and for assessing the presence of different and relevant contaminant microbial groups, all three samples were plated on different growth media, - plate count agar (PCA) to enumerate total aerobic mesophilic bacteria, manitol salt agar (MSA) to enumerate total *Staphylococcus*, rose bengal chloraphenicol agar (RBCA) to enumerate yeast and moulds, *Bacillus cereus* agar (BCA) to enumerate total *Bacillus cereus*, Violet red bile glucose agar (VRBGA) to enumerate total *Enterobacteriaceae*, de Man, Rogosa and Sharpe (MRS) agar to enumerate *Lactobacillus* and M17 agar to enumerate lactic streptococci. EF1 and EF2 samples were plated directly without any preparation; in contrast, Gr samples were soaked in a sterile saline solution (10 g/100 ml) before plating. The samples were then serially diluted in 0.1% peptone water, and the plates incubated aerobically at 37 °C for 3 days, at which time the number of colonies was counted.

Based on the first screening results, we chose MRS and M17 as the best media to perform the remaining tests. To isolate LAB, a 0.1 ml aliquot of sample was plated on either MRS or M17 agar plates (Lab M Ltd., Bury, UK) after the appropriate serial dilution in 0.1% peptone water. Samples were incubated under both aerobic and anaerobic conditions at 37 °C for 3 days. For the anaerobic experiments, a GasPak EZ Anaerobe Container System was used (Becton, Dickinson and Company, Baltimore, MD). When bacterial growth was observed, the colonies were counted and successively purified. After isolation, the bacteria were stored at –80 °C in 20% glycerol as a cryoprotective agent. Overall, 49 and 25 LAB were isolated on MRS agar and M17, respectively. Taking into account the higher number of isolates obtained on MRS agar, all further work was performed using this medium only.

Initial LAB characterization

To group the isolates, we first performed a preliminary characterization involving Gram staining and microscopic analyses (to determine morphology). We then tested all isolates for the ability to produce catalase and oxidase enzymes. The catalase test was performed by adding a few drops of hydrogen peroxide (3%) to freshly grown bacteria colonies; the formation of gas bubbles indicates a positive result for the test. The oxidase test was done by smearing a loop-full of colony of each isolate on BBL™ agar (Becton, Dickinson and Company) and observing possible colour changes.

Test for potential probiotic isolates

Selection of acid- and bile salt-tolerant isolates

For the acidity resistance test, selected isolates were grown in MRS broth which was acidified to pH 2.5 using concentrated hydrochloric acid. A preliminary screening assay was performed in a 96-well, flat bottomed microplate (NUNC, Roskilde, Denmark), using a microplate reader (FLUOstar, OPTIMA; BGM Labtech, Ortenberg, Germany). A 350- μ l sample of the inoculated media (inoculum concentration 1%) was placed in each well, and bacterial growth was followed by measuring the optical density of the solution at $\lambda = 620$ nm for 24 h at 37 °C. For each isolate, three replicates were performed. As a positive control, the isolates were grown in standard non-acidic MRS medium.

The acid-resistant strains were successively tested to assess their resistance to bile salt. The experiment was performed using the same experimental apparatus and protocols described for the acidity resistance test, with the exception that bile salt was added to the MRS broth (0.25 w/v).

Additional growth experiments were performed with those isolates which showed growth in acidic and bile salt medium, using the colony counting method to assess the extent of growth, in order to better evaluate and quantify the resistance of these isolates in these environments and to enable a better comparison of the growth in the different MRS media (unmodified and modified ones). Isolates were grown overnight at 37 °C. Tubes containing either acid or bile salt MRS medium were inoculated and incubated at 37 °C. At selected times (2, 4, 6, 8 and 24 h), 50- μ l aliquots were removed from the solution, properly diluted and plated on MRS agar. After incubation at 37 °C for 24 h, the grown colonies were counted. Each experiment and sampling were performed in triplicate, and growth was described as the average of the three values \pm standard deviation.

Test of antimicrobial activity

The antibacterial activity of those LAB isolates tolerant to conditions of high acidity and high bile salt concentration was tested. LAB isolates were grown in MRS broth at 37 °C for 24 h, following which the fully grown cultures were centrifuged (3000 g, 4 °C, 20 minutes; Rotina 35R centrifuge; Hettich Instruments, Tuttlingen, Germany). The supernatant was separated and sterilized by passage through a 0.2 μ m membrane filter (Whatman, Sigma-Aldrich, St. Louis, MO). The sterilized supernatant was then tested against seven indicator pathogenic microorganisms, namely, Methicillin-resistant *Staphylococcus aureus* (MRSA) (LMG 15975), *Salmonella enteritidis* (ATCC 3076), *Bacillus cereus*, *Escherichia coli* (ATCC 8739), *E. coli* (O157), *Listeria monocytogenes* and *Yersinia enterocolitica*.

The antibacterial activity of the LAB isolates against these pathogens was tested using the agar well diffusion method, as modified by Schillinger and Lucke (1987). Each pathogenic strain was grown on Mueller–Hinton plates for 24 h at 37 °C; from these plates, a liquid inoculum was prepared in a 0.85% saline solution, with an approximate colony concentration of 10^7 – 10^8 CFU/ml. A 5 ml aliquot of the inoculum was then spread homogeneously on the surface of a Mueller–Hinton agar plate using a sterile swab. A well was made into the plate using a sterile pipette (diameter 5 mm), and 30 µl of the LAB supernatant was then placed into each well, following which the plates were incubated at 37 °C for 24 h. After this time, the diameter of the inhibition halo around the well was measured. Each LAB strain was tested in triplicate, and the average diameter of the halo was calculated \pm SD. Pure MRS broth was used as negative control. Statistical analysis was performed on the results, and a multiple comparison analysis was used to compare the inhibition effect of the different isolates on the same pathogen (analysis of variance test, $p < 0.01$).

The strains which showed antibacterial activity were then further tested to determine which antimicrobial compound may have caused such activity. The experiments were performed using the same agar well diffusion method, but the LAB supernatant was modified before being tested in three different ways, as follows:

- The pH of the LAB supernatant was adjusted to 6.5 with 1 M NaOH in order to check if the activity was due to the acidity of the supernatant.
- 30 µl of catalase (5 mg/ml) was added to the LAB supernatant in order to determine whether the inhibitory effect was due to the presence of hydrogen peroxide.
- 30 µl of trypsin (9 mg/ml) was added to the LAB supernatant in order to see if the antibacterial agents were bacteriocins.

Molecular identification and typing of selected LAB isolates

Genomic DNA extraction

The selected LAB isolates were grown overnight in an appropriate liquid media and pelleted by centrifugation at maximum speed for 5 min. The pellets were then washed twice with TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). The total genomic DNA of the isolated strains was extracted using the guanidium thiocyanate–*N*-lauroylsarcosine denaturing method (Pitcher et al. (1989). The quantity and the purity of the total DNA was verified by agarose electrophoresis, and the DNA was stored at -20 °C until further use.

rDNA amplification and sequence data

rDNA gene sequencing data for the isolates were obtained for the approximately 1500-bp 16S rDNA region extending from nucleotide positions 27 to 1492 (*Escherichia coli* 16S rRNA gene sequence numbering) using the primers 27 F (3'-GAGT TTGATCCTGGCTCAG-5') and 1492R (3'-TACC TTGTTACGACTT-5') (Brosius et al. 1978). PCR assays were performed in an automated temperature cycling device (Techne Inc., Princeton, NJ), using 5 µl of total DNA, 25 µl NzyTaq 2× Green Master Mix (Nzytech, Lisbon, Portugal) and 2 µl of each primer in a total volume of 50 µl. The amplification cycling programme consisted of a 5-min initial denaturation at 94 °C, followed by 35 cycles of a 2-min denaturation at 94 °C, a 1-min annealing at 51 °C and a 2-min extension at 72 °C, with a final extension at 72 °C for 5 min. After the amplified fragments were verified by electrophoresis, they were purified and sequenced by Magrogen (Korea). Sequences were manually proofread, and nBLAST searches were performed using the GenBank Internet server (<http://www.ncbi.nlm.nih.gov>), for comparison with other strains deposited in the public databases, to identify the species taxon of each isolate. Sequences that showed more than 98% similarity were considered as belonging to the same taxonomy unit. The sequences obtained were deposited to the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) to allow public access.

RAPD-PCR typing

Genomic DNA from different strains were employed as templates for RAPD-PCR typing, using as a primer the M13 (Huey and Hall 1989) minisatellite core sequence (5'-GAGG GTGGCGGT TCT-3'). The amplification reactions contained 5 µl total DNA, 25 µl NzyTaq 2× Green Master Mix (Nzytech) and 2 µl of M13 primer in a total volume of 50 µl. The amplification cycling programme consisted of a 5-min initial denaturation at 94 °C, followed by 40 cycles of a 2-min denaturation at 94 °C, a 1-min annealing at 40 °C and a 2-min extension at 72 °C, with a final extension at 72 °C for 7 min. The PCR products were separated on 1.5% (w/v) agarose gels electrophoresis (1.5 V cm^{-1}) using SmartLadder (Eurogentec, Liège, Belgium) as DNA molecular weight marker and stained with GreenSafe Premium (Nzytech).

Results

Isolation of LAB strains

The effluents EF1 and EF2 (see Fig. 1a, b) and the final food product *gari* (Gr) were tested for the presence of LAB. *Fufu* itself was not tested, as it is likely that the strains isolated from

this food item would be similar to those isolated from *gari*, because there are no significantly different steps in the processing of the two food items which could affect the diversity of the microbial community.

Table 1 shows the pH of the three samples and the bacteria concentration measured on each growth medium. All samples had a low pH (3.1–3.4). The data in Table 1 also show that the bacterial growth was more enhanced in some media (i.e. PCA, MRS and M17) than in others. This was expected since media such as MRS and M17 are known to be the most appropriate ones for the growth and isolation of LAB (Oguntoyinbo and Narbad 2012). However, significant and similarly lower cell numbers were also observed in BCA, MSA and RBCA media, corresponding to the growth of *Bacillus*, *Staphylococcus* and yeast, respectively.

Subsequent screening on only MRS and M17 agar resulted in the isolation of 47 and 25 strains, respectively, confirming that microorganisms can be successfully isolated from these food sources. This result is in agreement published reports of strains being isolated from other spontaneously fermented African cereals and tubers (Okereke et al. 2012; Oyetayo 2010; Ukeyima et al. 2010).

As the highest number of isolates was observed to grow in MRS, this medium was chosen for further analyses. The isolates were tested to determine their morphology and Gram staining, as well as their response to the catalase and oxidase tests. The results of all these tests are shown in Fig. 2. All isolates for all three sources were Gram positive. Some differences, however, were observed in morphology and in the results of the catalase and oxidase tests. More specifically, 12 of the strains isolated from the EF1 presented a *Lactobacillus* profile, with rod morphology and negative results in the catalase and oxidase tests. Three additional strains, however, showed the same rod morphology but were negative and positive in the catalase and oxidase tests, respectively, indicating that they resembled *Bacillus* spp. For EF2, 11 strains

Table 1 pH values and microbial counts on different growth medium for the cassava (*Manihot esculenta*, Crantz) by-products and food item

Sample ^a	pH	Experimental media ^b						
		PCA	MSA	RBCA	BCA	VRBGA	MRS	M17
EF1	3.3	6.45	3.67	4.15	3.25	No growth	6.35	7.87
EF2	3.1	7.38	3.75	3.97	2.44	No growth	7.52	6.78
Gr	3.4	6.44	4.32	4.41	3.57	No growth	6.14	7.65

Values in table are presented as pH values as microbial counts (log CFU/ml for EF1 and EF2, log CFU/g for Gr)

^a EF1, EF2, Effluents of the *gari* and *fufu* production processes, respectively; Gr, *gari* food item

^b PCA, Plate count agar; MSA, manitol salt agar; RBCA, rose bengal chlorophenicol agar; BCA, *Bacillus cereus* agar; VRBFA, Violet red bile glucose agar; MRS, de Man, Rogosa and Sharpe agar

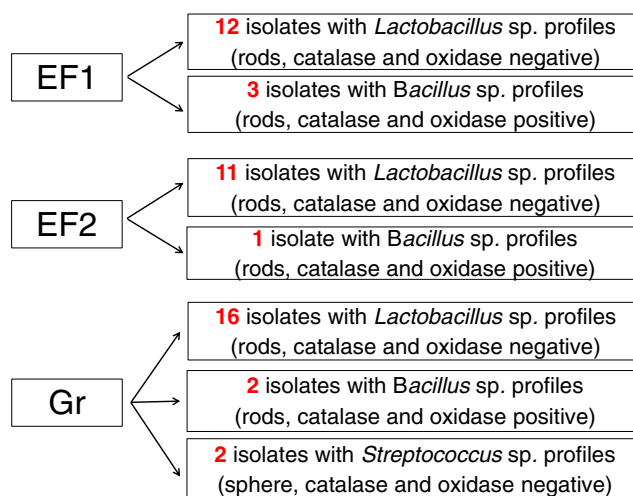


Fig. 2 Description of the isolates (all Gram-positive) from the different sources

presented the characteristics of *Lactobacillus*, while just one appeared to be *Bacillus* spp. For Gr, 16 and two isolates showed characteristic features of *Lactobacillus* and *Bacillus* spp., respectively; a further two isolates resembled *Streptococcus* spp., showing a spherical morphology and having negative results in both the catalase and oxidase tests.

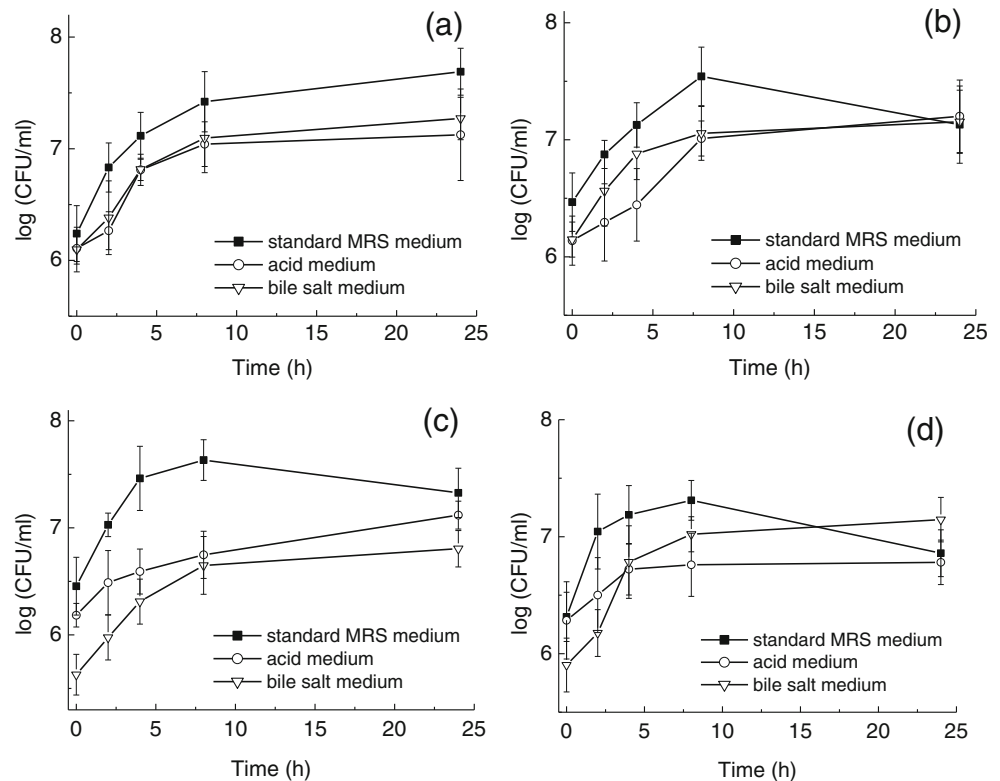
After these first screenings, we focused on those rod-shaped microorganisms which tested negative in both the catalase and oxidase tests as these characteristics were considered to be typical of LAB strains.

Tolerance to acidic pH and bile salt

Those strains which were identified to possess the desired characteristics were tested to determine their tolerance low pH (acidity) and bile salts, both essential properties for the isolates to be used as probiotics. To test tolerance to bile salts, the isolates were grown on media containing a bile salt concentration of 0.25% (w/v), as the mean intestinal bile concentration is 0.3% (w/v), although it may vary in different parts of the human gastrointestinal tract (Prasad et al. 1998). In these preliminary tests, bacterial growth, monitored by measuring the optical density of the culture solution, of four of the 41 strains tested showed good resistance to pH and bile salts, with relevant growth observed in both modified media (results not shown). More specifically, strain EF1.120 isolated from the EF1 samples, strain EF2.91 isolated from EF2 samples and strains Gr.20 and Gr.21 isolated from the Gr samples showed good growth.

These isolates were then tested for growth profiles in each media using the colony count method, the results of which are shown in Fig. 3. Although all four LAB isolates showed good growth, some differences between them were observed. Colonies of isolates EF1.120, EF2.91 and Gr.21 (Fig. 3a, b,

Fig. 3 Bacterial growth curves for selected isolates in standard de Man, Rogosa and Sharpe agar (MRS) agar and acidified and bile salt-containing media, respectively. **a** Strain EF1.120 isolated from EF1, **b** strain EF2.91 isolated from EF2, **c** strain Gr.20 isolated from Gr, **d** strain Gr.21 isolated from Gr. Symbols represent the average value \pm standard deviation (SD)



d, respectively) showed comparable growth values (within experimental error) for the whole monitored period in all three media, indicating that these isolates were barely affected by the acid or bile environment. In contrast, Gr.20 (Fig. 3c) showed relatively lower bacterial growth (about 1 order of magnitude lower) during the first hours of the curve, although by the end of the 24-h culture period, growth in acid or bile media is comparable to that in the unmodified MRS medium. This behaviour can be explained by the need for some LAB to adapt to the different conditions during the lag phase.

Overall, all of the tested isolates showed very good resistance profiles to the simulated hostile conditions of the gastrointestinal tract, indicating that they have the potential to be used as probiotics.

Antimicrobial spectrum of isolates

The antimicrobial properties of the selected isolates against several pathogenic bacteria were tested using the agar diffusion method; the average diameters of the halo (inhibition) zones are shown in Fig. 4a, b for Gram-negative and -positive strains, respectively. Based on the presence of halos, all Gram-negative pathogens tested were inhibited by the isolates, although the antimicrobial properties varied among the strains. *Escherichia coli*, for example, was more susceptible to EF2.91 than to the other isolates, but strain EF2.91 was less effective towards *Y. enterocolitica*. All four isolates showed a comparable

effect against *E. coli* (O157) (no significant difference observed), while strain Gr.20 was much less effective than the others towards *S. enteritidis*. The most susceptible strains were *S. enteritidis* and *Y. enterocolitica*, based on the diameter of their inhibition halos, which were as large as 26 mm. No pattern could be identified in the activity of the isolates, as they all showed very different behaviours depending on the pathogen considered. Isolate Gr.20, for example, was very effective towards *Y. enterocolitica* but much less against the other pathogens, while isolate Gr.21 showed high efficacy against *S. enteritidis* and *Y. enterocolitica* but a much lower efficacy against the two other strains.

Overall the Gram-positive strains showed more resistance to the isolates than did the Gram-negative ones (Fig. 4b). MRSA, for instance, showed an inhibition halo comparable to the diameter of the well when isolates EF2.91 and Gr.21 were used (5 and 6 mm, respectively). The other two pathogens, *B. cereus* and *L. monocytogenes*, were more susceptible, with *L. monocytogenes*, in particular significantly inhibited in a similar manner by all four isolates (no statistical difference observed between isolates). The antimicrobial activity of the LAB isolates against the Gram-positive pathogens was similar to that against Gram-negative ones in that there was no clear trend, with the antimicrobial activity of the LAB isolates differing according to the pathogen tested.

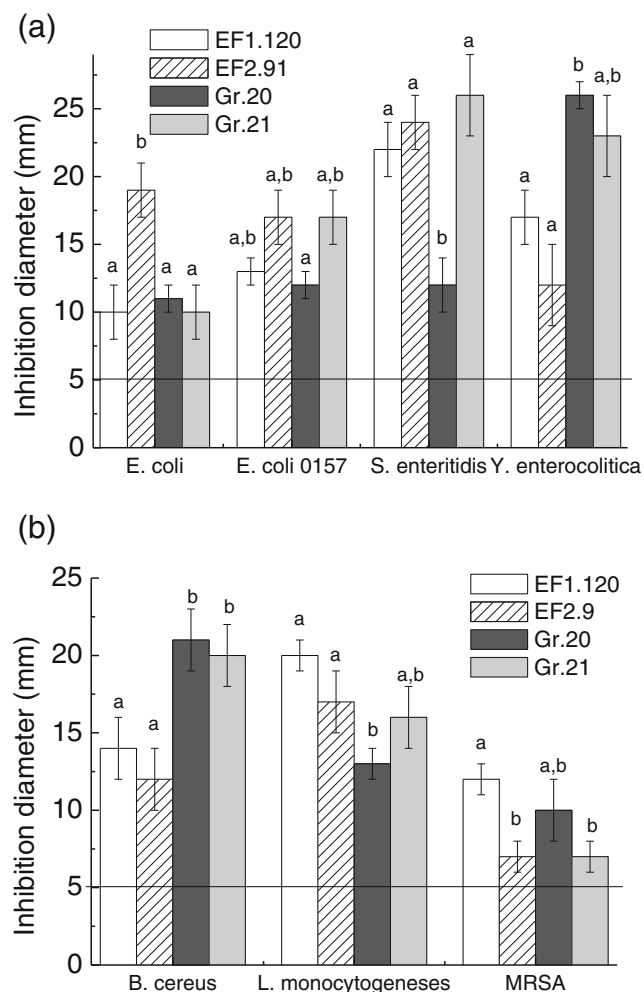


Fig. 4 Diameter of the inhibition zones (mm) around agar wells containing the respective isolates. **a** Gram-negative pathogenic bacteria, **b** Gram-positive pathogenic bacteria. Line at 5 mm represents the dimension of the well. Values are an average of three replicates \pm standard deviation. Different lowercase letters for the same pathogenic strain indicate a significant difference between the values (analysis of variance test, $p < 0.01$)

Antagonistic properties of the LAB isolates

Table 2 shows the antagonistic properties of the isolates; this test was performed to investigate the components of the metabolite responsible for the antimicrobial effects.

The tests performed with the neutralized supernatants of the isolates revealed that, in most cases, the supernatants retained their antagonistic properties against the pathogenic strains, with only MRSA no longer inhibited by either isolate EF1.120 or GR.20. This results leads to the conclusion that for MRSA, the high acidity of the isolates was partly responsible for the inhibition. Supernatants treated with catalase almost always still showed antibacterial activity, even if to a lesser extent (i.e. diameter of inhibition halo was smaller), with only

strain EF2.91 losing completely its activity towards both *E. coli* (O157) and *S. enteritidis*. In contrast, following the trypsin treatment, almost all isolates lost their antibacterial activity against all pathogenic strains; the only exceptions were strain Gr.20 towards *E. coli*, strain Gr.21 against *E. coli* (O157) and *S. enteritidis*, where some activity was still observed, even if much reduced.

These results indicate that the major contribution to the antibacterial properties of the isolates may be due to the presence of bacteriocins; at the same time, however, the supernatant acidity and the presence of hydrogen peroxide may also play a role.

Molecular identification of LAB isolates

The four isolates selected for further testing (EF1.120, EF2.91, Gr.20, Gr.21) were identified using the molecular biology methodologies described in section [Molecular identification and typing of selected LAB isolates](#). 16S rDNA gene sequencing revealed all four isolates to be *Lactobacillus fermentum* strains (confidence degree $E = 0.0$, homology of between 99 and 100% for all). The 16S rDNA sequences obtained were deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) under accession numbers KY041857 for strain EF1.120, KY041993 for strain EF2.92; KY042022 for strain Gr.20 and KY042021 for strain Gr.21.

The RAPD-PCR typing profiles, however, showed some differences, as can be seen in Fig. 5, indicating intra-specific differences. Indeed, the identification of different *L. fermentum* types has been reported in food and/or food-derived items (Papalexandratou et al. 2013; Wu et al. 2012). Figure 5 also shows some profile similarities between bacteria which were isolated from the same source. In fact, the profile of strain Gr.20 shares much more resemblance to that of Gr.21 than to those of EF1.120 and EF2.91, suggesting that the *L. fermentum* types present in *gari* were more similar, while quite different ones were present in the two effluents.

Discussion

As seen in Table 1, all tested samples had an acidic pH. This is expected, since lactic acid fermentation has been reported to take place during the processing of both *gari* and *fufu* (Anyogu et al. 2014). Because of this, the microbial community in this medium is expected to be preferentially LAB, as the bacteria must have developed adaptive mechanisms to the acidic conditions.

The fact that more strains were isolated on MRS agar than in M17 media can be explained by considering that MRS has been reported to be the more appropriate medium for lactobacilli growth, while M17 favours the growth of streptococci (Oguntoyinbo and Narbad 2012). Therefore, these data

Table 2 Antagonist properties of the supernatants of the isolates against the pathogenic bacteria tested

Pathogenic strains	Isolate ^a	Untreated supernatant	Neutral pH	Catalase-treated	Trypsin-treated
<i>Escherichia coli</i> (ATCC 8739)	EF1.120	+++	+	+	-
	EF2.91	+++	++	+	-
	Gr.20	+++	+	+	+
	Gr.21	+++	++	+	-
<i>E. coli</i> (O157)	EF1.120	+++	+	+	-
	EF2.91	+++	+	-	-
	Gr.20	+++	+	+	-
	Gr.21	+++	+	+	+
<i>Salmonella enteritidis</i>	EF1.120	+++	+	+	-
	EF2.91	+++	+	-	-
	Gr.20	+++	+	+	-
	Gr.21	+++	+	+	+
<i>Yersinia enterocolitica</i>	EF1.120	+++	+	+	-
	EF2.91	+++	+	+	-
	Gr.20	+++	+	+	-
	Gr.21	+++	+	+	-
<i>Bacillus cereus</i>	EF1.120	++	+	++	-
	EF2.91	++	+	+	-
	Gr.20	++	+	+	-
	Gr.21	++	+	+	-
<i>Listeria monocytogenes</i>	EF1.120	++	+	+	-
	EF2.91	++	+	+	-
	Gr.20	++	+	+	-
	Gr.21	++	+	+	-
Methicillin-resistant <i>Staphylococcus aureus</i>	EF1.120	++	-	+	-
	Gr.20	+++	-	+	-

-, No zone of inhibition; +, zones of inhibition of <7 mm in diameter; ++, zones of inhibition of between 8 and 11 mm; +++, zones of inhibition of >11 mm

^a Strain EF1.120 was isolated from EF1, strain EF2.91 was isolated from EF2, strains Gr.20 and Gr.21 were isolated from Gr

indicate that the tested samples contained more lactobacilli-type strains than streptococci-type strains. Such features (i.e. the composition of the microbial population) are not easy to predict for spontaneously fermented samples; in fact, the fermentation conditions and the sample final characteristics (i.e. pH, etc.) can selectively favour the survival of some strains more than others (Fayemi and Ojokoh 2014; Obinna-Echem et al. 2014).

The resistance to an acidic environment shown by the isolated strains is in agreement with data published in the literature. Indeed, previous published data show that LABs isolated from different sources are resistant to acidic media (Argyri et al. 2013; Haller et al. 2001; Kuda et al. 2014). In some cases, however, isolates do not survive in very acidic environments (pH of <3; Zamfir and Grosu-Tudor 2014). LAB resistance to bile salt has also been reported in the literature (Haller et al. 2001), and in some cases, a non-complete resistance has been reported for incubation times of between 3 and 6 h

(Solieri et al. 2014), similar to that observed in our study for sample Gr.20 (Fig. 3c).

Antibacterial activity is an important and desirable property for probiotic microorganisms, as a reduced growth of pathogens in the large intestine can lead to a decrease in gastroenteritis and food poisoning (Chapman et al. 2011). Previous studies have reported LAB isolated from fermented food which show antibacterial properties towards Gram-positive and Gram-negative strains (Grosu-Tudor et al. 2014; Iranmanesh et al. 2014); in particular, the antimicrobial activity of *L. fermentum* (i.e. the isolates identified here) has been reported (Archer and Halami 2015; Heredia-Castro et al. 2015).

The inhibition of the pathogenic strains by the LAB isolates in our study could be explained in the context of the high acidity of the isolates, due to the small chain fatty acids and lactic acid associated with LAB; however, the presence of either hydrogen peroxide or bacteriocin in the supernatants

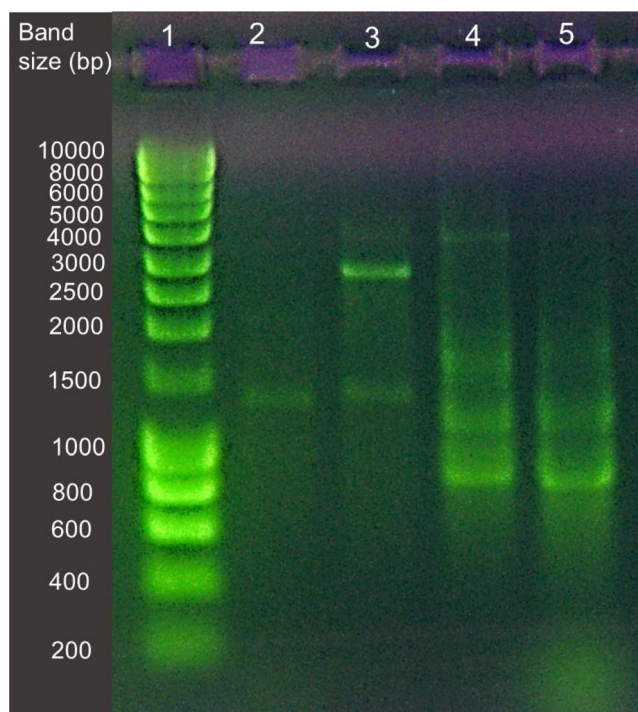


Fig. 5 Randomly amplified polymorphic DNA-PCR profiles of selected lactic acid bacteria isolates obtained with primer M13 amplification. Lanes: 1 SmartLadder molecular weight marker, 2 strain EF1.120, 3 strain EF2.91, 4 strain Gr.20, 5 strain Gr.21

of the cell-free isolates may also be relevant (Cabo et al. 2002). Bacteriocin antibacterial properties are well known and have been previously reported on (Grosu-Tudor et al. 2014; Hwanhlem et al. 2014; Iranmanesh et al. 2014; Ouwenand 1998), and many authors have particularly highlighted bacteriocin efficacy against pathogenic bacteria even at very low concentrations (Jimenez-Diaz et al. 1993; Klaenhammer and Kullen 1999; Vandenberg 1993). Examples of activity towards both Gram-positive and Gram-negative strains can be found in the relevant literature. For example, nisin and plantaricin 35d, both produced by *Lactobacillus plantarum*, were observed to be active against *Aeromonas hydrophila* (Messi et al. 2001). Other examples include bacteriocin ZJ008, produced by *Lactobacillus plantarum*, active against *Staphylococcus* spp. (Zhu et al. 2014) and bacteriocins produced by *Lactobacillus paracasei* subsp. *paracasei*, active against *Escherichia coli* (Caridi 2002). Thermophilin, produced by *Streptococcus thermophiles*, is also a powerful bacteriocin, with reports that it shows activity against *E. coli*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Gram negative), as well as *Bacillus* spp., *Listeria monocytogenes* and *Salmonella typhimurium* (Gram positive) (Ivanova et al. 1998).

All isolates in our study were identified as *Lactobacillus fermentum*, which has previously been isolated in cassava-derived products; as such, the data reported here are in agreement with published data (Anyogu et al. 2014; Banwo et al.

2012; Wilfrid Padonou et al. 2009). It has to be highlighted, however, that our study is the first to report the isolation of *L. fermentum* from cassava by-products, such as their effluents. The presence of *L. fermentum* strains in the effluents can be related to the presence of several different specific compounds (i.e. endogenous microflora) in the fermentation slurry. The mechanism(s) by which these compounds may favour LAB growth is not fully understood. One possibility is that these compounds act as an additional energy source for the bacteria. A connection between low pH and bacteria survival has also been reported (Obadina et al. 2006).

Conclusions

Based on the data derived from our study, we conclude that:

- Effluents from cassava processing can be considered a good source of LAB. The use of effluents from *gari* and *fufu* processing led to the isolation of different species of *L. fermentum*; other strains, however, could be isolated from different effluents.
- The isolates showed resistance in both acidic and bile salt media; moreover, they also demonstrated antibacterial activity towards both Gram-positive and Gram-negative pathogenic microorganisms.
- These features make the isolates suitable candidates for probiotic use. For their potential to be fully exploited, however, their safety should be tested; this will be indeed done in the future.

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