Title: Evaluation of the functional potential of *Weissella* and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow´s intestine.

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ABSTRACT

The characterization of 24 lactic acid bacteria (LAB) isolates from Nigerian traditional fermented dairy foods, including some cow´s intestine isolates, was conducted in order to select isolates for potential use as probiotics. LAB isolates were identified by partial sequencing the 16S rRNA gene as belonging to the species *Lactobacillus paracasei*, *Lactobacillus brevis* and mainly *Weissella confusa*. At the end of a characterization process, 2 *L. paracasei* and 2 *W. confusa* isolates were selected, and their resistance to a simulated gastrointestinal digestion and their ability to adhere to eukaryotic cell lines was assessed. The survival to the simulated gastrointestinal passage was higher when bacterial suspensions were made in skimmed milk (2.0±0.8 log units reduction) or at the simulated gastric juice pH 3 (2.7±0.9 log units reduction) than at pH 2.0 (5.5±0.7 log units reduction). Adhesion of LAB to both intestinal and vaginal epithelial models was comparable or higher than that of the reference *Lactobacillus rhamnosus* GG. However, some of the isolates increased the adhesion of the pathogen *Escherichia coli* LMG2092 to HT-29 and HeLa monolayers. Overall, isolates *L. paracasei* UI14 and *W. confusa* UI7 are good candidates for further studying potential benefits that support their use as probiotics. This is one of the few articles reporting the characterization and the probiotic potential of *Weissella*, although more studies are needed in order to establish their safety for potential probiotic applications.

Keywords: *Weissella*, *Lactobacillus*, probiotic, antibiotic resistance, gastrointestinal transit, adhesion epithelial cell culture

1. Introduction

Lactic acid bacteria (LAB) are being empirically employed from ancient times for the natural bio-preservation of raw materials. The development of food technologies involves the utilisation of specific, well-identified, and characterised LAB isolates to improve the safety as well as the organoleptical, nutritional or health properties of foods, i.e this is the basis for the concept of "functional starter" (Leroy and De Vuyst, 2004). Due to the long history of use in human consumption, some genera of LAB are "Generally Recognized As Safe" (GRAS) by the United States Food and Drug Administration (FDA). Several species of these genera are also included in the list of taxonomic units proposed by the European Food Safety Authority (EFSA) for "Qualified Presumption of Safety" (QPS) status. Among LAB with QPS status, those commonly found in fermented foods are *Lactococcus lactis, Streptococcus thermophilus*, and several species of *Lactobacillus, Leuconostoc* and *Pediococcus* (EFSA, 2007).

Some specific LAB strains are considered as probiotics, which have been defined by the WHO/FAO as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (WHO/FAO, 2006). The genera most commonly used as probiotics for oral delivery in human consumption are *Bifidobacterium* and *Lactobacillus* (Margolles et al., 2009). Even if some species of these genera are GRAS or have the QPS status, the working groups of the WHO/FAO recommend a proof that a given probiotic strain is safe. To check the safety, a series of *in vitro* tests are required such as, among others, the determination of the antibiotic resistant patterns and the production of toxic compounds. According to the guidelines proposed by these Organisations, one of the criteria for the selection of probiotic strains is their ability to transiently colonise the human mucosa (WHO/FAO, 2006). This property could help to maintain or improve the health of the intestinal and vaginal environments and thereby the well-being of the consumer (Abad and Safdar, 2009; Lee and Salminen, 2009).

In developed countries, the search for new strains with functional properties is of great interest from both health and industrial points of view. In this way, the traditional fermented foods from non-industrialised countries constitute a reservoir to search for new strains with novel functional properties (Lee et al., 2005; Mathara et al., 2004; Mohammed et al., 2009; Thapa et al., 2006). From the point of view of developing countries, the study of the properties of the isolated strains could contribute to improve the safety, quality and industrial value of traditionally fermented foods and could also open the possibility of their use for different applications.

In a previous work, we have reported the antimicrobial ability of isolates from Nigerian fermented foods and animal intestines against clinical pathogens strains obtained from patients with urinary tract infections (Ayeni et al., 2009). The aim of the current work was to identify and characterise these LAB and select from them some with probiotic potential through the study of their ability to survive to simulated gastrointestinal digestion, to adhere to epithelial cell lines and to inhibit the adhesion of *Escherichia coli* LMG2092.

2. Material and methods

2.1. Identification of isolates and growth conditions

In this study, we used 24 LAB isolates (Table 1) from different dairy products [cheese, fermented skimmed milk (nono), whey and milk] and animal sources (cow's large intestine) in four geographic regions of western Nigeria (Ekiti, Osun, Ondo and Oyo). They were selected from 134 initial isolates using as a criterion their ability to inhibit the growth of uropathogenic *Staphylococcus aureus* strain (Ayeni et al., 2009). Selected LAB were grown in MRS (Biokar Diagnostics, Beauvais, France) broth for 24 h at 37ºC and 5% CO_2 in a Heracell® 240 incubator (Thermo Electron LDD GmbH, Langenselbold, Germany) and were spread on the surface of

MRS (Biokar) agar plates to check purity. A single colony was picked up to prepare new stocks (stored at -80ºC in MRS with 20% glycerol) which were identified by partially sequencing the 16S rRNA gene using the Y1-Y2 primers as previously reported (around 350 bp) (Ruas-Madiedo et al., 2005). As standard procedure, LAB isolates from stocks were cultivated overnight at 37ºC, 5% CO_2 and used to inoculate (2%) fresh MRS media which were cultivated for 24 h under the same conditions.

2.2. Production of lactic acid and volatile compounds

The production of lactic acid in the supernatants of 24 h grown LAB cultures was measured by ion-exchange HPLC using a chromatographic system composed of an Alliance 2690 module injector, a Photodiode Array PDA 996 detector and the Empower software (Waters, Milford, MA, USA) under conditions previously described (Ruas-Madiedo et al., 2005). The volatile compounds were determined by means of head-space (HS) GC-MS. Samples (400 µL) of supernatants were mixed with cyclohexanone (0.36 mg/mL) as internal standard and were placed into 10-ml glass tubes sealed with rubber and metallic caps. The analysis was carried out in a 6890N Agilent GC coupled with a HS automatic injector G1888 series and with a 5975B inert MS detector (Agilent Technologies Inc., Palo Alto, CA) using conditions previously reported (Salazar et al., 2009).

2.3. Antibiotic resistance pattern

The minimal inhibitory concentration (MIC) against several antibiotics was studied in 9 isolates selected according to their origin and belonging to different species. VetMic™ Lact-I microdilution tests (SVA, Uppsala, Sweden) were used to determine the MIC to gentamicin

(concentration range tested: 0.5-256 µg/mL), kanamycin (2-1024 µg/mL), streptomycin (0.5-256 µg/mL), neomycin (0.5-256 µg/mL), tetracycline (0.12-64 µg/mL), erythromycin (0.016-8 µg/mL), clindamycin (0.03-16 µg/mL), and chloramphenicol (0.12-64 µg/mL). Additionally, hand-made plates were used for the following antibiotics: ampicillin (1-1024 µg/mL, Apollo Scientific Ltd., Cheshire, UK), ciprofloxacin (0.1-128 µg/mL, Sigma Chemical Co., St. Luis, MO, USA), trimethoprim – sulfamethoxazole (TMP-SMX, 0.25-256 µg/mL, Celtech Pharma S.A., Madrid, Spain), fosfomycin (3.13-3200 µg/mL, Pharmazam, Barcelona, Spain) and nitrofurantoin (0.25-256 µg/mL, Laboratorios ERN S.A., Barcelona, Spain). When necessary, the concentration of the excipient was subtracted for the calculation of the corresponding concentrations of each antibiotic. The LAB isolates were cultured overnight on agar LSM [90% Isosensitest (Oxoid) and 10% MRS, (Klare et al., 2005)]. Individual colonies were suspended in a sterile glass tube containing 5 ml sterile saline solution. The density of each saline suspension was adjusted to an $OD_{625\ nm}$ of between 0.16 - 0.2 was obtained. The saline suspensions were then diluted 1:1000 to obtain a final concentration of about $3x10^5$ CFU/mL, which corresponds to the McFarland standard 1. Afterwards, 100 µL of the diluted suspensions were added to each well and incubated at 37°C for 48 h. Bacterial growth was visually detected as a pellet at the bottom of the well and also using a Microplate Spectrophotometer Benchmark Plus (BioRad, Hercules, CA, USA). The MIC was defined as the lowest antibiotic concentration at which no visual growth was detected. When it was possible, the breakpoint standard of the FEEDAP panel (EFSA, 2008) was used.

2.4. Probiotic selection criteria

2.4.1. Survival to the gastrointestinal transit

135 The survival to the chemically simulated gastrointestinal (GIT) transit was studied for 4
136 LAB, previously selected base on their antibiotic susceptibility profile, their antagonistic activity
137 towards pathogens and their antimicrobial metabolite production (see supplementary material).
138 The GIT transit simulation was performed using the procedure described by Sánchez and co-
139 workers (2010). In brief, 24-h MRS grown cultures (around 7 log CFU/ml for *Weissella* and 9 log
140 CFU/ml for *Lactobacillus*) were washed twice in saline solution and resuspended in
141 gastrointestinal juice (GJ: 125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$ and 0.3% pepsin) at
142 different pH: 2.0, 3.0 and 2.0 containing 10% skim-milk (Difco®, Becton Dickinson, Franklin
143 Lakes, NJ, USA). After 90 min incubation at 37ºC in aerobic conditions, bacterial suspensions
144 were centrifuged, resuspended in duodenal juice (DJ: 1% bovine bile, pH 8.0) and incubated for
145 10 min at 37ºC in an anaerobic chamber (10% H_2, 10% CO_2 and 80% N_2). In the final step,
146 bacterial suspensions were collected and resuspended in intestinal juice [IJ: 0.3% bovine bile
147 (Sigma), 0.1% porcine pancreatin (Sigma), pH 8.0] and incubated for 180 min under anaerobic
148 conditions previously indicated. Experiments were carried out in triplicate and in all steps viable
149 counts were obtained by plating in MRS agar. The final percentage of survival was calculated
150 from the ratio: CFU recovered bacteria after complete GIT challenge / CFU initial bacteria. For
151 counting, serial dilutions in Ringer (Merck, Darmstadt, Germany) solution were made and they
152 were pour-plated on MRS agar which was incubated for 48 h at 37ºC, 5% CO_2.
153
154 *2.4.2. Adhesion to epithelial intestinal and vaginal cell lines*
155 Three epithelial intestinal cell lines, Caco-2, HT-29 and HT-29-MTX, the last one kindly
156 supplied by Dr. T. Lesuffleur (Lesuffleur et al., 1990), as well as the epithelial vaginal cell line
157 HeLa were used to assess the adhesion ability of the 4 selected bacteria. The culture and
158 maintenance of the cell lines were carried out following standard procedures (Sánchez et al.,

159 2010) using DMEM medium for Caco-2, HT-29-MTX and HeLa, and McCoy´s medium for HT-
160 29. All media were supplemented with 10% foetal serum bovine and with a mixture of antibiotics
161 (50 µg/mL penicillin, 50 µg/mL streptomycin, 50 µg/mL gentamicin and 1.25 µg/mL
162 amphotericin B). Media and reagents were purchased from Sigma. The cell lines were used after
163 reached the confluent differentiated stay (13±1 days). The strain *Lactobacillus rhamnosus*
164 LMG18243 (also named GG) was used as positive control of well-recognised adherent LAB.
165 Cultures of the five LAB, grown overnight in 10 ml MRS under standard conditions, were
166 centrifuged, washed twice in Dulbecco´s PBS solution (Sigma) and resuspended in DMEM or
167 McCoy´s media without antibiotics at a concentration of about 10^8 CFU/mL. Cell line
168 monolayers were also washed twice in Dulbecco´s PBS, to remove antibiotics, and bacterial
169 suspensions (10^8 CFU/ml) were added at a ratio bacteria: eukaryotic cell of 10:1. Plates were
170 incubated for 1 h at 37°C, 5% CO_2 and, afterwards, wells were gently washed three times with
171 Dulbecco's PBS buffer to remove the non-adhered bacteria. Then, monolayers were trypsinized
172 with EDTA-trypsine solution (Sigma) and bacterial counts were carried out in MRS agar.
173 Adhesion results were expressed as the percentage of bacteria adhered with respect to the amount
174 of bacteria added (% CFU bacteria adhered / CFU bacteria added). Two replicated experiments
175 were carried out for each isolate using two independent microplates (each isolate tested by
176 duplicate in each plate).
177
178 *2.4.3. Competitive exclusion of* Escherichia coli *adhesion to epithelial cell lines by probiotics*
179 The ability of the 4 selected LAB isolates to *in vitro* compete with *E. coli* LMG2092 to
180 adhere to intestinal and vaginal epithelia, was tested using the cell lines HT-29 and HeLa,
181 respectively (Collado et al., 2007). LAB were grown for 24 h in MRS, as previously described,

and *E. coli* LMG2092 was cultured overnight in LB medium at 37ºC under rigorous shaking. Bacterial cultures were washed with Dulbecco´s PBS and resuspended in the corresponding cell line media without antibiotics at concentration about 10^8 CFU/mL. Afterwards 500 μL of each LAB were mixed with the same volume of *E. coli* (ratio LAB: *E. coli* about 1:1) and added to each cell line type (ratio bacteria: eukaryotic cell about 10:1). Incubation took place for 1 h at 37ºC in at atmosphere containing 5% CO_2 (v/v). Subsequently, counts of *E. coli* were made in agar-LB containing 5 μg/mL erythromycin (24 h of incubation at 37ºC, 5% CO_2). This erythromycin concentration was higher than the MIC detected in the four LAB isolates selected (Table 2) and checked experimentally. *E. coli* adhesion results were expressed as the percentage of *E. coli* adhered with respect to the amount added. The adhesion percentage of strain LMG2092 in the absence of LAB was also tested. Two replicated experiments were carried out for each isolate using two independent microplates (each isolate tested by duplicate in each plate).

2.5. Statistical analysis

Data of GIT challenge and bacteria adhesion were statistically analysed using the SPSS 11.0 software for Windows (SPSS Inc., Chicago IL, USA) by mean of independent one-way ANOVA tests. For bacterial GIT-resistance, the differences were tested at the final step after the sequential GIT challenge, using as factor "gastric juice type" with three categories "pH 2.0, pH 3.0 and pH 2.0+skim-milk". For LAB adhesion, the factor was "LAB type" with five categories "UI6, UI7, UI14, UI22 and GG". For *E. coli* adhesion, the factor was "Bacteria type" with five categories "UI6, UI7, UI14, UI22 and LMG2092". In all cases, the mean comparison LSD (least-significant difference, $p<0.05$) test was used to assess differences among categories of each factor.

3. Results

3.1. Identification and characterisation of LAB

The 24 isolates from dairy sources (whey, milk, nono and cheese) and cow´s intestine were identified at species level by partially sequencing the 16S rRNA gene (Table 1). Most of the isolates belonged to the species *Weissella confusa* (71%) followed by *Lactobacillus paracasei* (21%) and *Lactobacillus brevis* (8%). The growth characteristics of the isolates in MRS broth (supplementary material table 1) varied amongst the different isolates. Table 1 shows that lactic acid was the major organic acid produced by these bacteria during growth, ranging from 13 to 21 mg/mL depending on the isolate, being slightly higher in those of the *L. paracasei* species. The volatile compounds profile found in the supernatants of 24 h cultures was also representative of each species (Table 1). Thus, *W. confusa* and *L. brevis* produced similar levels of ethanol (higher than 3 mg/mL) and acetaldehyde, varying accordingly to isolate. In contrast, *L. paracasei* cultures produced very low amounts of ethanol (ranging from 10 - 26 μg/mL) and, in general, they synthesised higher amount of acetone and lower level of acetaldehyde than the other two species. To select isolates for further analyses of their probiotic potential, other parameters such as production of H_2O_2 and bacteriocin-like metabolites, as well as antimicrobial activity against five entero-pathogens (supplementary material tables 1 and 2) were also investigated for the 24 LAB isolates. The presence of genes involved in the synthesis of biogenic amines was used as exclusion criterion and the capability to grow in milk was considered as a positive technological characteristic for potential strain delivery in dairy products. Nine isolates were selected on the basis of their origin and species identification, as well as their technological, antimicrobial and safety performance. The isolates *L. paracasei* UI14 and UI22, *L. brevis* UI3 and UI12, and *W.*

228 *confusa* UI4, UI6, UI7, UI18 and UI21 were chosen for further characterisation of their antibiotic
229 resistance profiles.
230
231 *3.2. Resistance to antibiotics profile*
232 Table 2 shows the MICs of the 9 selected LAB isolates. In general, lactobacilli did not
233 display high resistance levels to aminoglycosides (gentamicin, kanamycin, streptomycin and
234 neomycin), erythromycin, clindamycin and ampicillin. On the contrary, resistance to tetracycline
235 in *L. brevis*, in *L. brevis* and *L. paracasei* were close the suggested breakpoints for these species
236 and that of chloramphenicol for some strains was even above (EFSA, 2008). Regarding *W.*
237 *confusa* isolates, we can point out the extraordinarily high sensitivity of *W. confusa* UI6 to
238 aminoglycoside antibiotics, in comparison to the other four isolates. In contrast to *L. paracasei*
239 and *L. brevis*, all *W. confusa* isolates were resistant to TMP-SMX (MIC > 256 μg/mL). Finally,
240 all the isolates tested from both genera were highly resistant to fosfomycin and nitrofurantoin. In
241 this step, a final selection was done in which *L. brevis* isolates were excluded due to the presence
242 of tetracycline resistance, in addition to the presence of genes codifying biogenic amines
243 (supplementary material table 1). *W. confusa* UI4, UI18 and UI21 were not included in the final
244 selection due to their kanamycin, streptomycin and/or neomycin resistance (Table 2). Thus,
245 isolates *L. paracasei* UI14 and UI22, as well as *W. confusa* UI6 and UI7 were selected for further
246 study their probiotic potential.
247
248 *3.3. Survival to simulated gastrointestinal transit*
249 If a putative probiotic strain is intended for oral delivery, it should survive the adverse
250 conditions of the upper GIT tract to arrive alive to the target action site, the small and large

intestine. The viability losses of the 4 selected *L. paracasei* and *W. confusa* isolates along the different steps of the simulated GIT transit is depicted in Fig. 1. All isolates were sensitive to the simulated GJ pH 2.0, with viability losses up to 6 log units. In all cases, the addition of 10% skimmed milk to GJ pH 2.0 (which raised the pH near 4.0), or the use of GJ pH 3.0 attenuated these decreases in the viability and, besides, differences in simulated DJ (containing 1% w/v bovine bile) became apparent; *W. confusa* UI7 and *L. paracasei* UI14 resisted better the high bile salt concentration, showing lower viability decreases than the other two isolates. The final challenge to the IJ (containing 0.3% bovine bile and pancreatin) did not affect the viability of isolates. Overall, viability decrease (respect to the initial values) was improved for all isolates if the initial pH of GJ was adjusted to 3.0 or the GJ pH 2.0 was supplemented with 10% skim milk whereas, survival percentages of isolates under pH 2.0 challenge were lower than 0.003%. In addition, the statistical analysis of bacterial survival after exposure to sequential GIT transit simulation (Table 3), showed that the highest survival percentages ($p < 0.05$) were those of isolates *W. confusa* UI7 and *L. paracasei* UI14.

3.4. Adhesion to epithelial cell lines and pathogen inhibition

One of the methods for estimating the ability of probiotics to transiently colonize the human mucosa is the *in vitro* quantification of the adhesion capability to representative cell lines. In our case, the adhesion of the 4 selected isolates to the epithelial intestinal cell lines Caco-2, HT-29 and HT-29-MTX, as well as to the epithelial vaginal cell line HeLa was evaluated (Fig. 2). Overall, adhesion of the four selected isolates to Caco-2 and HT-29 cell monolayers was poor if compared with the positive control *L. rhamnosus* GG. Adhesion percentages were better when HT-29-MTX was used as matrix, being in some cases even better than that of *L. rhamnosus* GG (isolates *W. confusa* UI7 and *L. paracasei* UI14, $p < 0.05$). In addition, *L. paracasei* isolates,

275 especially UI14, showed similar or better adhesion than strain *L. rhamnosus* GG to HeLa cell
276 monolayers, whereas the adhesion of the *Weisella* isolates to this cell line was lower.
277 Finally, the ability of the 4 LAB isolates to competitively inhibit the adhesion of *E. coli*
278 LMG2092 to HT-29 or HeLa cell monolayers was also estimated (Fig. 3). In general, none of the
279 LAB isolates inhibited the adhesion of the pathogen to both epithelial cell lines; even in some
280 cases the opposite effect was detected. For the vaginal HeLa cell line, the isolate *L. paracasei*
281 UI22 clearly favoured the adhesion of the pathogen, whereas the other three isolates increased the
282 *E. coli* adherence to the intestinal cell line HT-29.
283
284 **4. Discussion**
285 In the present study, 24 LAB isolates from Nigerian dairy products and cow´s intestine
286 were studied and, after a selection process, the probiotic potential of two *L. paracasei* and two *W.*
287 *confusa* isolates was assessed. The most abundant isolates, identified by partial 16S rDNA gene
288 sequencing, belonged to the genus *Weissella*, which is frequently isolated from different
289 traditional fermented foods (Lee et al., 2005; Scheirlinck et al., 2007; Thapa et al., 2006) and,
290 specifically, the species *W. confusa* is present in some fermented vegetables and milk (Björkroth
291 et al., 2002), as well as in human faeces of healthy individuals (Walter et al., 2001). *Weissella*
292 species are also common inhabitants of the vaginal microbiota (Jin et al., 2007; Silvester and
293 Dicks, 2003) and even a strain of *Weissella kimchii* has been proposed as probiotic to prevent
294 vaginal infections (Lee, 2005). However, the safety of this genus has not been deeply studied, and
295 thereby, its application as probiotic should be taken carefully. Two species of *Lactobacillus* were
296 identified in this study, *L. paracasei* and *L. brevis*. This genus is naturally associated with a large
297 variety of nutritive-rich plant and animal environments and from these origins it can be present as
298 contaminant in the raw materials used for traditional food fermentations, such as milk, meat,

299 vegetables and cereals (Margolles et al., 2009). In addition, several species of this genus,
300 including *L. brevis* and *L. paracasei*, are included in the list of taxonomic units proposed by the
301 EFSA for QPS status (EFSA, 2007). That is, based in the current knowledge available, they are
302 safe for human consumption and, in fact, *L. paracasei* is present in many commercial probiotic
303 dairy foods (Gueimonde et al., 2004). Nevertheless, the safety assessment of every strain should
304 be checked for food use (WHO/FAO, 2006).

305 After the first step of a rational selection process, the antibiotic resistance profiles were
306 obtained for a subset of 9 isolates. It has been reported that resistance of LAB to antibiotics is
307 more pronounced in sub-Sahara Africa than in other parts of the world because of the
308 indiscriminate use of antibiotics both in human and animals (Olukoya et al., 1993). In these
309 circumstances it is extremely important to know and characterize the resistance to antibiotics in
310 strains potentially intended for food and/or therapeutic applications. The MIC against several
311 antibiotics of the selected LAB showed that, in general, the *Lactobacillus* isolates did not display
312 high resistance levels to most antibiotics. However, the high resistance to tetracycline in *L. brevis*
313 and to chloramphenicol in *L. brevis* and *L. paracasei* suggested that specific antibiotic resistance
314 genes could be responsible for the resistance phenotype to these two antibiotics (EFSA, 2008;
315 Huys et al, 2008). This implies that the presence of transferable resistance genes should be
316 experimentally analysed as the basis for the resistance, before potential food applications of these
317 strains. All isolates tested were highly resistant to nitrofurantoin. In this regard, high resistance
318 levels to this antibiotic have been previously reported in lactobacilli (Charteris, 1998). In relation
319 to *W. confusa* isolates, data on antibiotic resistance profiles are much scarcer. There are no
320 defined breakpoints and just a few studies have dealt with this point (Ouoba et al., 2008; Vay et
321 al., 2007). In spite of that, it is important to point out the high sensitivity of *W. confusa* UI6 to
322 aminoglycosides, which is likely due to an increased intake of the antibiotic by the bacteria, or to

323 a facilitated binding to the ribosome. A similar phenotype has been reported in other LAB
324 (Korhonenen al., 2008). Regarding chloramphenicol, resistance levels were similar to those found
325 for lactobacilli. Although there are no defined breakpoints for this genus, this result could
326 indicate the presence of specific chloramphenicol resistance genes in *Weissella*, which deserves
327 future studies before application of these isolates in foods. Interestingly, and in contrast to *L.*
328 *paracasei* and *L. brevis*, all *W. confusa* isolates were resistant to TMP-SMX (MIC > 256 μg/mL).
329 This fact seems to be quite common in *Weissella* species (Liu et al., 2009) and could indicate that
330 microorganisms of this genus posses an intrinsic resistance to antibiotics that inhibit
331 tetrahydrofolate biosynthesis. Finally, the high resistance levels to fosfomycin detected in the
332 three species were similar to that previously described in some other Gram-positive bacteria
333 (Arca et al., 1997). *Weissella* species are common inhabitants of the vaginal microbiota and
334 TMP-SMX is used as one of the main therapies for genitourinary tract infections (David et al.,
335 2005). Thus, the *W. confusa* isolates characterized in this work could be used to balance the
336 normal vaginal microbiota during or after antibiotic treatments. It is controversial whether strains
337 of *W. confusa* could be used as human probiotics for oral delivery in foods, since in spite of being
338 detected in human faeces (Walker et al., 2001), it is not clear the relevance of this genus in the
339 complex microbiota community or its interaction with the host. Additionally, safety is another
340 issue that needs to be addressed before the proposal of *Weissella* as probiotic for human use.
341 Although, it is worth mentioning that this genus was defined around 1990, including previous
342 species *Leuconostoc paramesenteroides*, *Lactobacillus confusus*, *Lactobacillus halotolerans*,
343 *Lactobacillus kandleri*, *Lactobacillus minor* and *Lactobacillus viridescens*, and (at that decade) a
344 novel species *Weissella hellenica*, and that members of current *Weissella* genus have been
345 consumed by humans from a variety of traditional fermented products (Bjorkroth et al., 2002).

346 Five out of the 9 selected isolates were excluded due to their antibiotic profiles and the
347 other 4 were selected for their probiotic characterization using some of the *in vitro* tests proposed
348 by the WHO/FAO Organizations (WHO/FAO, 2006). One of the criteria for the selection of a
349 given strain as probiotic is its ability to survival to the stressing conditions of the human GIT
350 tract, mainly low pH in the stomach and bile salts in the small intestine (Sánchez et al., 2008).
351 The acidic pH stress conferred by the GJ, affected the viability of the 4 selected isolates in a
352 lesser extent at pH 3 or if skimmed milk (10 % w/v) was included in the formulation. This fact
353 evidenced both the high intrinsic sensibility of the isolates to low pH and the protective effect of
354 skimmed milk, and agrees with our previous findings with *Bifidobacterium animalis* subsp. *lactis*
355 (Sánchez et al., 2010). Surprisingly, intrinsic resistance of the selected isolates to bile was better
356 than expected, and in fact moderate viability losses at the end of the simulated GIT digestion
357 were detected for *L. paracasei* UI14 and *W. confusa* UI7 with an initial GJ adjusted to pH 3, or
358 with a GJ adjusted to pH 2 in the presence of skimmed milk. It is thought that a good survival to
359 bile may reflect also a good resistance of the probiotic to the adverse conditions of the human
360 GIT tract (Watson et al., 2008). For this reason, the selected isolates could be proposed for oral
361 delivery if a probiotic effect is demonstrated. In this regard, oral delivery is a convenient method
362 for both intestinal and vaginal probiotic targets. It has been proved that orally ingested probiotics,
363 in combination with antibiotic therapy, could restore the normal microbiota of the vagina since
364 this niche is re-colonised with the rectal microbiota (Anukam et al., 2006; Ozkinay et al., 2005).
365 Another criterion for selection of LAB recommended by the WHO/FAO guideline is their
366 ability to transiently colonise the human GIT or vaginal mucosa (WHO/FAO, 2006). Usually, the
367 adhesion capabilities of a given strain are studied using epithelial cell line monolayers, being
368 Caco-2, HT-29 and HeLa among the most used cell lines (Vélez et al., 2007). In the present
369 study, we have used *L. rhamnosus* GG, a probiotic bacterium that is commonly used as positive

370 control for adhesion (Tuomola and Salminen, 1998). In general, and in comparison with the
371 adhesion values obtained for the GG strain, the selected isolates presented lower adhesion to
372 Caco-2 and HT-29 cell monolayers, whereas its adhesion to HT-29-MTX was similar or higher.
373 The last one is a mucin-producing colonocyte-like cell line and it has been reported that many
374 LAB and bifidobacteria are able to stick this protective layer covering the intestinal mucosa
375 (Collado el al., 2007; Ruas-Madiedo et al., 2006). Similarly, *L. paracasei* isolates showed a
376 comparable or significantly better adherence to the vaginal HeLa cell line than strain GG. In
377 certain situations, LAB may form a biofilm on mucosa surfaces which could confer a physical
378 barrier for non-desirable microorganisms (Antonio et al., 2005). Globally, the good adhesion of
379 the selected isolates, mainly *L. paracasei* UI14, to both vaginal and intestinal epithelia supports
380 their potential use in both anatomic locations if a probiotic effect is shown.
381 In certain situations, opportunistic pathogens invade the human mucosa due to a decrease
382 in the number of protective bacteria. In this sense, exogenous probiotic administration may
383 inhibit pathogen adhesion by competitively blocking common receptor molecules. In addition,
384 some probiotics have antimicrobial capability which is normally due to the production of several
385 primary and secondary metabolites, such as organic acids (mainly lactic acid), carbon dioxide,
386 volatile compounds (ethanol, diacetyl), hydrogen peroxide and bacteriocins, among other factors
387 (Rouse and van Sinderen, 2008). Most isolates tested in this study increased the adhesion of the
388 pathogen *E. coli* LMG2092 to HT-29, as well as the isolate *L. paracasei* UI22 to HeLa cell
389 monolayers. This fact has been previously observed for some commercial probiotic strains
390 (Collado et al., 2007), as well as for lactobacilli and bifidobacteria from different origins
391 (Gueimonde et al., 2006; Ruas-Madiedo, 2006). Our results suggest that some LAB, seen as
392 receptor structures, might immobilize the pathogens thus blocking their adhesion to the
393 eukaryotic cells. This hypothesis needs to be demonstrated, but it has been proposed that the

394 formation of such a ternary complexes eukaryotic cell-LAB-pathogen could be able to confer an
395 antimicrobial effect since as far as LAB and pathogens stay in a close contact, the effect of
396 potential antimicrobials could be magnified (Boris and Barbés, 2000; Osset et al., 2001).
397 Moreover, it has been reported that a direct contact between probiotics and pathogens can
398 decrease the cell division rate of the latter (Coudeyras et al., 2009). In any case, the relevance of
399 the increase of *E. coli* adhesion to cell lines in presence of some of our LAB isolates deserves
400 further investigations.
401
402 Summarising, in the present study we have characterized 24 LAB and 4 of them were
403 further selected to *in vitro* evaluate their use as potential probiotics. In general, the isolates
404 showed good survival to the GIT transit and acceptable adhesion capability to intestinal and
405 vaginal epithelia when compared to their respective positive controls. *L. paracasei* isolates were
406 able to grow and acidify milk thus indicating good technological properties for the manufacture
407 of dairy fermented foods. Besides, *L. paracasei* species has the QPS status whereas *Weissella*
408 genus, which has been studied in less extent, for the moment was not included in the EFSA list.
409 Although this study aimed a preliminary characterization of the probiotic potential of the 4
410 selected strains, we could suggest that UI7 isolated, showing slightly better adhesion than the rest
411 of isolates to intestinal cell lines, could be most suitable for a potential application in the gut
412 ecosystem. For vaginal applications, UI14 could be the most interesting isolate. However, more
413 studies are needed mainly concerning safety aspects, and health benefit properties before the
414 application of these isolates to the formulation of functional probiotics intended for oral or
415 vaginal delivery.
416
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571

572 **Table 1**

573 Origin of LAB isolates from different traditional fermented dairy products and cow´s intestine[1].

				Mean ± SD			
				mg/mL	μg/mL		mg/mL
Species	Isolate	Origin	Region	Lactic acid	Acetaldehyde	Acetone	Ethanol
L. paracasei	UI1	Whey	Oyo	21.77±2.93	13.32±0.20	36.21±5.73	0.012±0.003
	UI2	Whey	Oyo	20.26±0.97	15.77±3.04	34.66±4.75	0.026±0.013
	UI9	Whey	Osun	19.74±0.88	12.99±2.94	32.06±4.45	0.024±0.015
	UI14	Whey	Oyo	19.68±0.71	12.77±2.43	38.06±5.54	0.019±0.006
	UI22	Whey	Ekiti	18.07±4.36	12.56±2.78	27.22±7.97	0.010±0.003
L. brevis	UI3	Whey	Oyo	16.54±3.45	42.43±9.11	3.99±0.14	3.27±1.22
	UI12	Cow intestine	Ekiti	13.70±3.80	24.87±2.42	7.48±4.57	4.29±0.37
W. confusa	UI4	Whey	Oyo	13.22±2.04	51.84±5.86	28.09±4.09	4.03±0.24
	UI5	Cow intestine	Oyo	13.39±1.02	46.73±7.02	23.88±3.73	3.81±0.28
	UI6	Cow intestine	Oyo	14.81±1.94	49.72±7.65	23.65±4.88	3.47±1.57
	UI7	Cheese	Oyo	13.61±1.78	45.71±10.06	23.07±0.42	3.78±0.12
	UI8	Whey	Ekiti	15.39±3.57	49.58±13.39	24.63±1.30	4.01±0.46
	UI10	Nono	Ondo	15.93±3.22	39.32±8.93	26.59±8.27	2.68±1.32
	UI11	Whey	Osun	13.37±1.32	56.56±12.06	30.33±4.86	4.82±0.61
	UI13	Whey	Ekiti	14.43±2.05	54.78±14.00	30.38±4.20	4.70±0.54
	UI15	Cow intestine	Oyo	14.14±2.17	39.07±6.23	28.30±1.55	3.96±0.21
	UI16	Milk	Ekiti	13.93±2.30	64.02±25.49	32.75±16.83	5.03±1.81
	UI17	Cow intestine	Oyo	15.00±2.73	49.95±14.15	26.49±1.32	4.13±0.50
	UI18	Cheese	Osun	14.26±2.28	46.76±14.35	24.81±4.55	4.00±0.54
	UI19	Cow intestine	Oyo	14.26±1.97	45.88±10.11	22.25±2.02	3.89±0.26
	UI20	Cow intestine	Oyo	15.01±2.32	44.92±9.08	16.91±1.14	4.07±0.16
	UI21	Milk	Ekiti	13.73±2.10	47.01±6.75	19.70±5.81	3.81±0.50
	UI23	Nono	Ondo	13.59±1.38	35.05±16.78	15.80±7.76	3.94±0.54
	UI24	Cow intestine	Oyo	14.14±2.49	55.90±15.70	21.17±0.50	4.91±0.83

574 1. Production of metabolites (lactic acid, acetaldehyde, acetone and ethanol) in MRS broth after 24 h of cultivation.

Table 2

Minimum inhibitory concentration (MIC) against antibiotics of selected LAB isolates from different sources.

Bacteria	Isolate	MIC (μg/mL)												
		GEN	KAN	STR	NEO	TET	ERY	CLI	CHL	AMP	CIP	TMP-SMX	FOF	NIT
L. paracasei	UI14	2	128	32	16	1	0.12	0.06	8	4	2	16	>3200	256
	UI22	2	32	32	4	1	0.12	0.06	4	4	2	8	>3200	256
L. brevis	UI3	<0.5	8	8	<0.5	16	0.12	0.25	4	4	8	2	>3200	>256
	UI12	<0.5	8	8	<0.5	16	0.12	0.25	8	4	8	4	>3200	>256
W. confusa	UI4	2	64	32	2	4	0.25	0.5	16	2	2	>256	>3200	>256
	UI6	<0.5	<0.2	1	<0.5	4	0.12	0.5	16	<1	<2	>256	>3200	>256
	UI7	8	128	64	8	8	0.25	0.06	8	8	4	>256	>3200	>256
	UI18	4	128	32	>256	4	0.5	0.12	8	4	2	>256	>3200	>256
	UI21	2	32	16	5	4	0.25	0.12	4	4	8	>256	>3200	>256

GEN: gentamicin, KAN: kanamycin, STR: streptomycin, NEO: neomycin, TET: tetracycline, ERY: erythromycin, CLI: clindamycin, CHL: chloranphenicol, AMP: ampicillin, CIP: ciprofloxacin, TMP-SMX: trimethoprim – sulfamethoxazole, FOF: fosfomycin, and NIT: nitrofurantoin.

583 **Table 3**
584 Percentage of survival after the chemically simulated gastrointestinal transit.
585

Sample [1]		Mean ± SD [2]	
		Isolates	% survival [3]
pH 2.0 + 10% milk	*W. confusa*	UI6	1.04±0.62 [a]
		UI7	4.13±0.87 [b]
	L. paracasei	UI14	1.39±0.86 [a]
		UI22	0.19±0.14 [a]
			**
pH 3.0	*W. confusa*	UI6	0.03±0.02 [a]
		UI7	2.11±0.11 [c]
	L. paracasei	UI14	1.22±0.88 [b]
		UI22	0.04±0.01 [a]
			**

586 1. The survival percentage for sample pH 2.0 was lower than 0.003% for all isolates.
587 2. CFU /mL recovered with respect to the initial number of bacteria
588 3. Values within each sample not sharing a common letter are significantly different ($p<0.05$)
589 according to the LSD (least significant difference) mean comparison test.
590 ** One-way ANOVA $p<0.01$
591

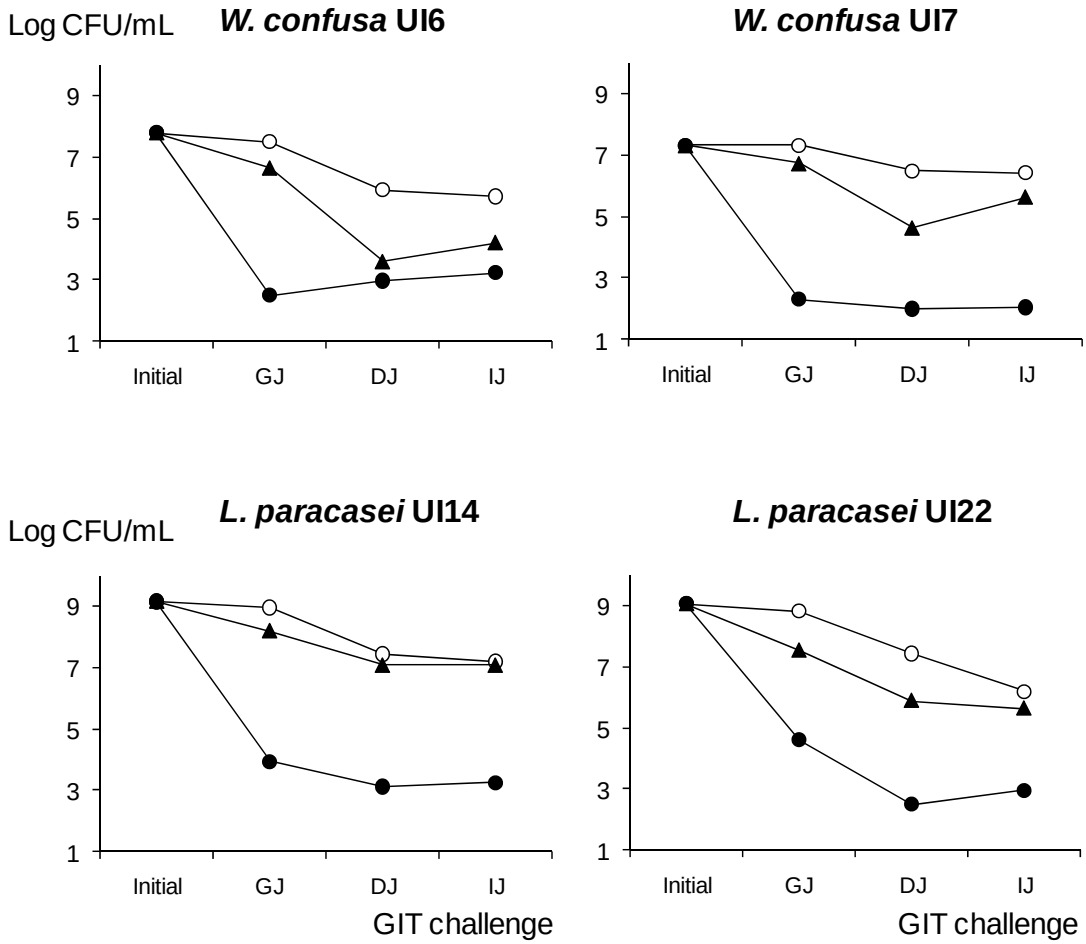
FIGURE LEGEND

Figure 1: Counts (Log CFU/mL) of four isolates of *Weissella confusa* and *Lactobacillus paracasei* after the chemically simulated gastrointestinal transit. GJ: gastric juice (pH 2.0 black circles, pH 2.0 + 10% skim-milk white circles, and pH 3.0 black triangles), DJ: duodenal juice and IJ: intestinal juice. The coefficient of variation (standard deviation*100/mean) of these results varied ranged among 1% to 12 %.

Figure 2: Percentage of adhesion (CFU/mL of adhered bacteria respect to CFU/mL of added bacteria) of four isolates of *Weissella confusa* and *Lactobacillus paracasei* to the intestinal epithelial lines Caco2, HT29 and HT29-MTX and to the vaginal epithelial line HeLa. Whiting each cell line, columns that do not share the same letter are statistically different according to the LSD (least significant differences) mean comparison test ($p < 0.05$). *Lactobacillus rhamnosus* GG was used as reference strain.

Figure 3: Inhibition of *Escherichia coli* LMG2092 adhesion to the intestinal epithelial line HT29 and to the vaginal epithelial line HeLa, in presence (and absence) of four isolates of *Weissella confusa* and *Lactobacillus paracasei*. The percentage of *E. coli* adhesion was calculated as follows: CFU/mL of adhered *E. coli* respect to CFU/mL of added *E. coli*. Within each cell line, columns that do not share the same letter are statistically different according to the LSD (least significant differences) mean comparison test ($p < 0.05$).

613 **Figure 1**
614



615
616

Figure 2

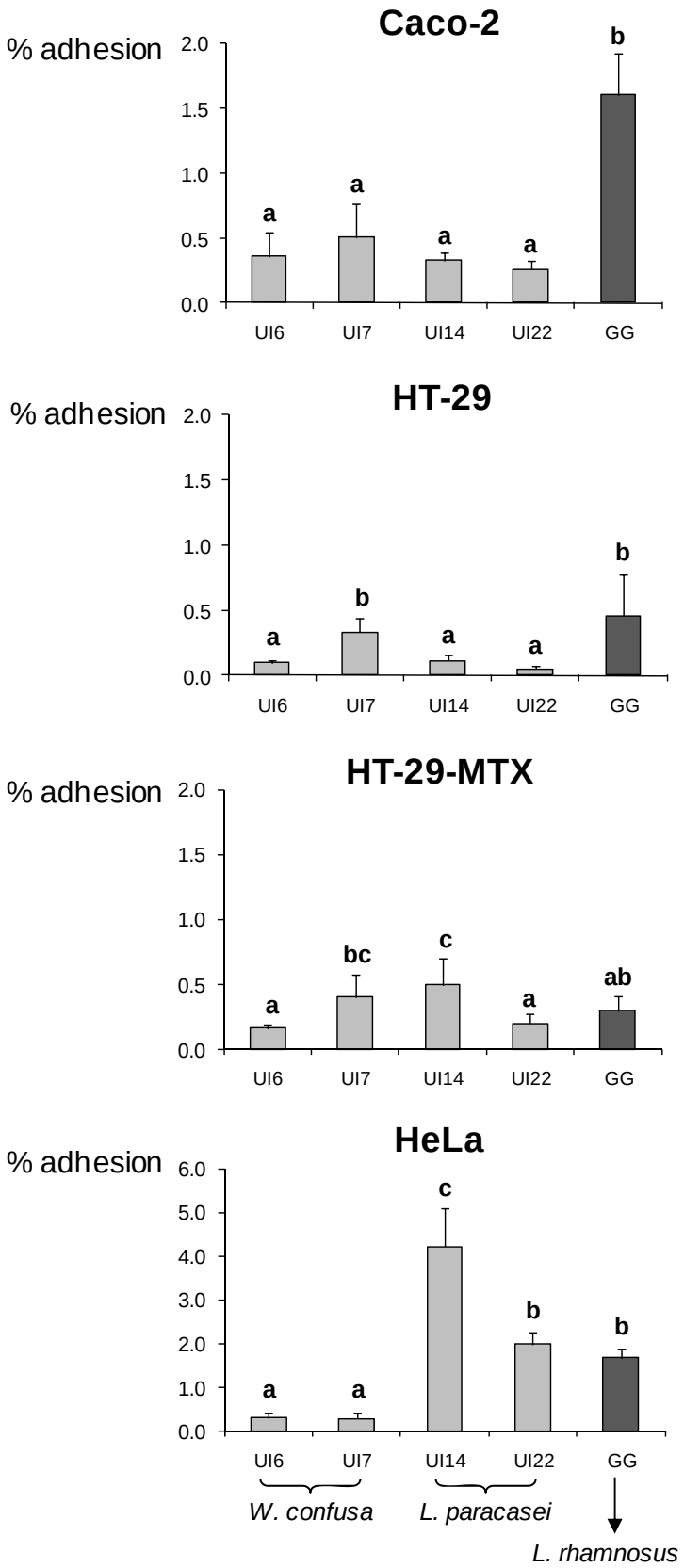
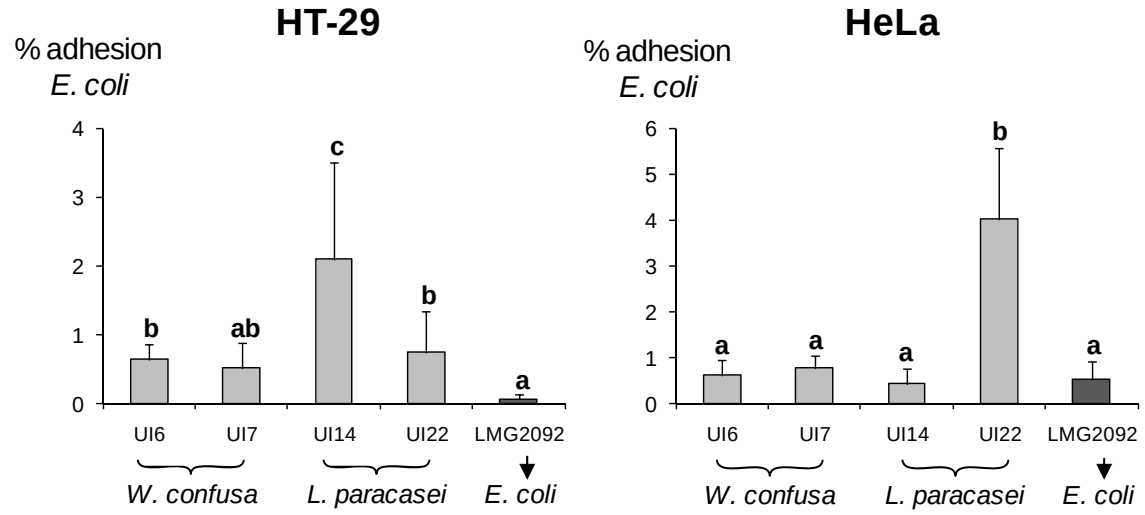

Caco-2
% adhesion
2.0
1.5
1.0
0.5
0.0
a
a
a
a
b
UI6
UI7
UI14
UI22
GG
HT-29
% adhesion
a
b
a
a
b
HT-29-MTX
% adhesion
a
bc
c
a
ab
HeLa
% adhesion
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
a
c
b
b
W. confusa
L. paracasei
L. rhamnosus

Figure 3


HT-29
% adhesion
E. coli
4
3
2
1
0
b
ab
c
b
a
UI6
UI7
UI14
UI22
LMG2092
W. confusa
L. paracasei
E. coli
HeLa
% adhesion
E. coli
6
5
4
3
2
1
0
a
a
a
b
a
UI6
UI7
UI14
UI22
LMG2092
W. confusa
L. paracasei
E. coli