Screening of lactic acid bacteria isolated from fermented table olives with potential probiotic

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Running title: Potential probiotic lactobacilli in table olives

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Abstract

The aim of this work was to study the potential probiotic properties of lactobacilli associated with table olives. From a total of 111 isolates from spontaneously fermented green olive brines, 109 were identified at species level by multiplex PCR amplifications of the *recA* gene. One hundred and seven of these were identified as *Lactobacillus pentosus*, one as *Lactobacillus plantarum*, and another as *Lactobacillus paraplantarum.* Repetitive bacterial DNA element fingerprinting (rep-PCR) with GTG_5 primer revealed a higher variability within the *L. pentosus* isolates and nine different clusters were obtained. Most of them showed high autoaggregation ability, low hydrophobicity properties, and lower survival to gastric than to pancreatic digestion; however, no isolate showed bacteriocin, haemolytic or bile salt hydrolase activities. A multivariate analysis based on results from phenotypic tests led to the segregation of some *L. pentosus* isolates with promising potential probiotic characteristics, which are even better than probiotic reference strains. Due to the autochthonous origin of the strains, their use as starter cultures may contribute to improving natural fermentation and the nutritional characteristics of table olives.

Keywords: table olives; lactic acid bacteria; molecular identification; starter; probiotic potential

1. Introduction

According to the latest consolidated statistics, worldwide table olive production for the 2007/2008 season was 2,153,500 tonnes (IOOC, 2008). Most table olives are prepared as Spanish-style or alkali treated green olives (IOOC, 2004), which represent about 60% of production, but there is also a general increasing interest for traditional and homemade products such as “seasoned” olives, usually prepared from directly brined fruits (Arroyo López et al., 2005).

Among the microorganisms involved in the Spanish style green table olive fermentation, lactic acid bacteria (LAB) is the most relevant group (Garrido-Fernández, Fernández Díaz, & Adams, 1997). The development of LAB in this process leads to a rapid acidification of brines and a drop in pH to below 4.5 (Garrido-Fernández et al., 1997; Sánchez, Rejano, Montaño, & De Castro, 2001; Delgado et al., 2007). However, the growth of LAB in directly brined olives is more difficult due to the high presence of phenolic compounds in the storage solutions. For this reason, LAB from non alkali-treated fruits have been scarcely studied (Garrido-Fernández et al. 1997).

According to FAO/WHO (2001), probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host". They “should be resistant to gastric juices and be able to grow in the presence of bile”. There is a series of *in vitro* tests such as acid and bile tolerance, antimicrobial production, etc, that, although requiring further refinement, are usually applied as a first approach for the selection of potential probiotic microorganisms (FAO/WHO, 2001).

Reviews on the importance of foods in the efficacy of probiotics have recently been published (Champagne and Gadner, 2005; Ranadheera, Baines, and Adams, 2010). New sources of probiotic microorganisms such as Greek dry-fermented sausage (Papamanoli,

Tzanetakis, Litopoulou-Tzanetaki, & Kotzekidou, 2003) cereal-based substrates (malt, barley and wheat media) (Charalampopoulos, Pandiella, and Webb, 2002) or kimchi (Chang, Shim, Cha, and Chee, 2010) are currently being investigated. The regular intake of probiotic preparations is considered useful to increase the bioavailability of trace minerals and proteins in the vegetarian diet (Famularo, De Simone, Pandey, Ranjan Sahu, & Minisola, 2005). Potentially probiotic strains of *Lactobacillus plantarum* and *Lactobacillus paracasei* survived in artichokes, which improved their survival in simulated gastrointestinal digestion (Valerio et al., 2006). *L. paracasei* IMPC2.1 (isolated from the human intestine) incorporated into an artichoke fed to a human was recovered from stools and also showed high viability for 3 months in ripe and green olive fermentations (Lavermicocca et al. 2005a). The use of table olives as a reliable food vehicle for this bacterium into the human gastrointestinal tract has been subjected to patents (Lavermicocca et al. 2005b; Hurtado, Reguant, Bordons & Rozès, 2012). Recently, *L. paracasei* IMPC2.1 has been used as starter culture for processing green table olives and its survival and dominance on the olive surface during fermentation was studied through molecular identification (De Bellis, Valerio, Sisto, Lonigro, & Lavermicocca, 2010). However, the probiotic potential of LAB naturally occurring in table olive fermentations still remains unexplored. The availability of LAB strains isolated from this product, the most common lactic acid fermented vegetable in western countries, could have a considerable value for developing starter cultures with the double role of being technologically relevant and able to enhance the health aspect of table olives.

Therefore, the present study focused on a) the identification and molecular characterization at strain level of the lactobacilli microbiota isolated from two types of table olive elaborations: lye-treated (Spanish style) and directly brined olives, and b) the study of certain *in vitro* phenotypic characteristics related to the probiotic potential of these microorganisms.

2. Materials and methods

2.1. Olive cultivars and processing

Samples were directly obtained from three Spanish olive factories. In the case of Spanish style green olive fermentations, a single factory (plant A, located in Alcalá de Guadaira, Seville, Spain), which processes both Gordal and Manzanilla cultivars in underground, fiberglass containers with a 10-tonne capacity, was sampled. Briefly, Gordal olives were first treated with a 20 g ml^{-1} sodium hydroxide solution until reaching 2/3 of the flesh, washed with tap water for 18 h and then immersed in 110 g ml^{-1} of an NaCl brine. Manzanilla olives followed the same treatment, except that the sodium hydroxide solution was 25 g ml^{-1}. For directly brined olives, two factories which ferment Gordal and Manzanilla fruits (plant B, Sanlucar la Mayor, Sevilla, Spain) and Aloreña cultivar (plant C, Alhaurín el Grande, Málaga, Spain) were visited. Both factories used plastic drum containers with 500-kg capacity. In this case, olives were washed and directly brined in 110 g ml^{-1}, 80 g l^{-1} and 70 g l^{-1} of NaCl solutions for Aloreña, Gordal and Manzanilla cultivars, respectively. Manzanilla and Gordal containers were kept in an open yard whereas Aloreña containers were maintained in a cold room (~8 ºC) and also in a covered yard at ambient temperature. None of the three sampled factories had previously used LAB starter cultures. All fermentations were allowed to evolve spontaneously. For each factory and type of process, at least two fermentation containers were analyzed.

2.2. Fermentation control

Physicochemical control of the fermentation was achieved through analyses of the pH, NaCl concentration (g ml^{-1}), titratable (lactic acid) and combined (undissociated organic salts) acidities, expressed as g ml^{-1} and mEq l^{-1}, respectively, at 0 (initial), 10, 20, 40, 60 and 105

days of fermentation. These parameters were analyzed according to the methodology described by Garrido-Fernández et al. (1997).

2.3. LAB isolation

Samples for microbiological purposes were taken at the same sampling times described above. Brine samples were diluted in sterile saline solution (9 g l^{-1} NaCl) and then plated using a Spiral System model dwScientific (Dow Whitley Scientific Limited, England) on de Man, Rogosa and Sharpe (MRS) agar (Oxoid) supplemented with 0.2 g l^{-1} sodium azide (Sigma, St. Luis, USA) as selective medium for LAB isolation. Plates were incubated at 30 ºC for 48 h and then the colonies were counted using a CounterMat v.3.10 (IUL, Barcelona, Spain) image analysis system and expressed as $\log_{10}$ CFU ml^{-1}.

A total of 111 isolates were randomly selected during the different stages of fermentation, 80 from Spanish style green olives, 28 from Gordal and Manzanilla directly brined olives and 3 from Aloreña directly brined olives. Then, cultures were purified by subsequent re-streaking on MRS agar and observed under a phase contrast microscope (Olympus Optical Co., LTD Tokyo, Japan) to differentiate cell morphology.

2.4. Molecular identification and characterization of the lactobacilli isolates

The different isolates grown on MRS agar supplemented with sodium azide were identified at species level using multiplex PCR analysis of the *rec*A gene with species-specific primers for *L. pentosus*, *L. plantarum* and *L. paraplantarum*, following the protocol described by Torriani, Felis, and Dellaglio (2001). Previously, DNA was extracted from 1 ml of an overnight culture of each isolate according to the procedure describe by Andrigetto, Zampese, and Lombardi (2001), with the addition of lysozyme (50 mg ml^{-1}, Sigma) for cell lysis.

Then, rep-PCR fingerprinting was performed for molecular strain characterization with the single oligonucleotide primer $(GTG)_5$, following the protocol described by Gevers, Huys, and Swings (2001). Products of rep-PCR were electrophoresed in a 20 g l^{-1} agarose gel and the profiles obtained were visualized under ultraviolet light using UVI pro platinum 1.1 Gel Software (Eppendorf, Hamburg, Germany). The resulting fingerprints were analyzed with the BioNumerics 4.6 software package (Applied Maths, Kortrijk, Belgium). The similarity among digitalized profiles was calculated using the Pearson correlation and an average linkage (UPGMA) dendrogram was derived from the profiles.

2.5. In vitro phenotypic tests related to probiotic potential

Autoaggregation assays were performed according to the methodology described by Kos et al. (2003) with slight modifications. Four ml of an overnight culture containing ~10^8 CFU ml^{-1} were homogeneized by vigorous vortexing for 10 s and then incubated at room temperature for 5 h. At 1 h intervals, 0.1 ml of the upper suspension were carefully removed, transferred to a new tube containing 3.9 ml of phosphate buffered saline (PBS), and then the absorbance at 600 nm (A_{600}) was measured in a SmartSpec™3000 (BIORAD Laboratoires, USA). The autoaggregation percentage was expressed as a function of time until it was constant, using the formula $1-(A_t/A_0) \times 100$, where A_t represents the absorbance at any time (1, 2, 3, 4 or 5 h), and A_0 the absorbance at time t=0 h.

Bacterial cell surface hydrophobicity was assessed by measuring microbial adhesion to hydrocarbons using the procedure described by Crow, Gopal, and Wicken (1995). Briefly, cells at the stationary phase were centrifuged (10,000 x *g*, 5 min). The resulting pellet was washed twice in PBS, re-suspended in 3 ml of 0.1 mol l^{-1} KNO_3 (final concentration ca. 10^8 CFU ml^{-1}) and the A_{600} was measured (A_0). One ml of xylene was then added to the cell suspension to form a two-phase system. After a 10 min pre-incubation at room temperature,

the two-phase system was mixed by vortexing for 2 min. Then, the water and xylene phases were separated by incubation for 20 min at room temperature (~25 ºC). The aqueous phase was carefully removed and the A_{600} was measured (A_1). The percentage of the cell surface hydrophobicity (H%) was calculated using the formula $H\% = (1-A_1/A_0) \times 100$.

For the detection of bacteriocin production, the agar well diffusion assay test was used. A lawn of BHI soft agar (10 g l^{-1},) medium containing each indicator microorganism, namely *Salmonella enteritidis* ATCC13076 and the strain named as WT, a food isolate, *Listeria monocytogenes* FMCC B-128 and NCTC 10527, and *Escherichia coli* ATCC 35150 and NCTC 12079, was poured onto Petri dishes. After solidification, a hole was made in the center of the plate and 100 µl of a 36-48 h cell-free MRS broth of the different lactobacilli strains were placed in ithe hole and allowed to diffuse at 4°C for 30 min. After an overnight incubation at 37°C, the plates were examined for halos around the hole.

To test for haemolytic activity, fresh lactobacilli broth cultures were streaked onto Columbia agar plates containing 50 g l^{-1} of horse blood (Oxoid, Milan, Italy), and incubated for 48 h at 30 ºC. Then, the plates were examined for signs of α, β or γ-haemolysis.

The ability to deconjugate bile salts was tested by using the plate assay described by Dashkevicz and Feighner (1989). Briefly, bile salt–MRS agar plates containing 5 g l^{-1} of Oxgall (Sigma-B3883) were inoculated with an overnight MRS culture, incubated at 37 ºC for 72 h, and then observed for colonies with precipitated bile salts (BSH activity). If present, the diameters of the precipitate halos around the colonies were measured.

Simulated gastric digestion (SGD) was studied using the protocol described by Corcoran, Stanton, Fitzgerald, and Ross (2007) with slight modifications. Simulated, synthetic gastric juice was prepared in a buffer solution at pH 2.0 containing NaCl (2.05 g l^{-1}), KH_2PO_4 (0.60 g l^{-1}), $CaCl_2$ (0.11 g l^{-1}) and KCl (0.37 g l^{-1}). It was adjusted to pH 2.0 with 1 M HCl and autoclaved at 121 ºC for 15 min. Prior to use, pepsin (0.0133 g l^{-1}) and lysozyme

(0.01 g l^{-1}) were added. All the components were obtained from Sigma-Aldrich. The lactobacilli cultures to be tested were grown to early stationary phase, centrifuged (10,000 x *g*, 10 min) and the pellet was washed with the buffer (pH 2.0) mentioned above. Then, the cells were re-suspended in the synthetic gastric juice to a final concentration between 10^7 and 10^{10} CFU ml^{-1} and incubated for 3 h at 37 ºC in an orbital shaker (~200 rpm) to simulate peristaltic movements. Finally, serial dilutions of the cultures were plated onto MRS agar medium and counted after incubation at 30 ºC for 3-5 days.

Simulated pancreatic digestion (SPD) was formulated using bile (3 g l^{-1}, Oxoid) and pancreatin (0.1 g l^{-1}, Sigma) in a buffer at pH 8.0 consisting of 50.81 g l^{-1} of sodium phosphate dibasic heptahydrate and 8.5 g l^{-1} of NaCl. The pH was adjusted with a solution of potassium phosphate monobasic anhydrous at 1.27 g l^{-1}. Harvested cells from the previous gastric digestion step were washed in saline and re-suspended in the same volume of the simulated pancreatic juice. A colony count on MRS plates at time zero (T_0), to know the initial counts in the freshly prepared simulated pancreatic juice, was performed by serial dilutions of the cultures. After shaking at 200 rpm in an orbital shaker overnight at 37 ºC, the pellet was washed and then re-suspended in a volume of isotonic solution in order to avoid carryover of the buffers to the agar. Serial dilutions were done, and subsequently plated onto MRS agar. Reference probiotic strains *Lactobacillus casei* Shirota and *Lactobacillus rhamnosus* GG (kindly provided by Dr. Simone Guglielmetti from the University of Milan, Italy) were used for comparison of the survival rates after simulated gastric and pancreatic digestion. Overall, survival was obtained by comparison of the initial lactobacilli counts at the start of the simulated gastric digestion and those remaining at the end of the simulated pancreatic digestion.

2.6. Multivariate analysis

Lactobacilli activities were subjected to multivariate analysis to check their suitability to be analyzed by chemometric techniques. Principal Component Analysis (PCA) was used to discriminate among lactobacilli isolates, using a varimax rotation. For the selection of the number of Principal Components or factors, the Kaiser criterion mentioned by Jolliffe (1986) was followed, and only factors with eigenvalues higher than 1.00 were retained. The cases introduced in the analysis were the 109 identified lactobacilli isolates while the discriminating variables were hydrophobicity, auto-aggregation, gastric digestion survival, pancreatic digestion survival and overall digestion survival. Statistical analyses were performed with Statistic 7.0 software package (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Physicochemical changes

The initial and final NaCl concentrations, combined and titratable acidities, and pH values for the different table olive elaborations studied in this work are shown in Table 1. The initial NaCl concentrations in directly brined olives ranged from 59.5 g l^{-1} (Manzanilla) to 91.0 g l^{-1} (Aloreña) and decreased to ~20.0 g l^{-1} at the end of the process. In Spanish style olives, the NaCl concentration was kept constant at around 64.0 g l^{-1} throughout the process. Combined acidity was significantly higher in Spanish style compared to directly green brined olives, in which it increased markedly at the end of fermentation. The initial pH was around 4.5 in directly brined olives (because of the initial brine correction with acetic acid) while it was higher (~5.6) in the Spanish style. As expected, the fermentation produced a decrease in pH and an increase in titratable acidity at the end of fermentation, which was more pronounced in Spanish style olives.

3.2. LAB changes during fermentation

The changes in the LAB population in Spanish style green table olive fermentations showed the typical evolution for this type of processes (Figure 1). Initial counts were around 6 $\log_{10}$ CFU ml^{-1}, reaching a maximum population level of ~8 $\log_{10}$ CFU ml^{-1} at the second week of fermentation. Then, LAB counts decreased slowly but a population level of above 6 $\log_{10}$ CFU ml^{-1} was always present. In directly brined Gordal and Manzanilla olives, the initial LAB counts were much lower (below 2 $\log_{10}$ CFU ml^{-1}) and reached their maximum population levels (6 $\log_{10}$ CFU ml^{-1}) between the 3rd and the 4th week of processing. Then, the populations were kept constant until the end of fermentation. In Aloreña directly brined olives stored in a cold room, LAB were not detected during the period of study while in the drums placed in the yard at ambient temperature, they grew slowly after a very large lag phase and reached a population of around 5 $\log_{10}$ CFU ml^{-1} at the 20th week.

3.3. Molecular identification and characterization of the lactobacilli isolates

In the present survey, a total of 111 LAB isolates were subjected to PCR analysis of the *rec*A gene with species-specific primers for *L. pentosus*, *L. plantarum* and *L. paraplantarum. The* results obtained indicated that a total of 2 isolates (1.8%) could not be identified as belonging to any of these three bacterial species, 107 strains (96.4%) were identified as *L. pentosus,* one strain (0.9%) was identified as *L. plantarum* and another strain (0.9%) as *L. paraplantarum.* .These last two species were both isolated from Spanish style green olive fermentations.

The 107 *L. pentosus* isolates were then subjected to molecular characterization to define their intra-specific variability, which was successfully accomplished by means of rep-PCR using the GTG_5 primer (Gevers et al. 2001; Svec, Drab, & Sedlacek, 2005). As shown in Figure 2, by arbitrarily selecting a similarity coefficient of 80%, a total of 9 clusters could be identified. Among them, clusters VI and VII grouped 46 and 25 isolates, respectively,

representing 66% of the *L. pentosus* strains considered in this study. The other 7 clusters contained lower amounts of isolates, from 9 of cluster VIII to 2 of clusters II and V. It is worth noting that the composition of cluster VI was constituted mainly by isolates of plant A (42 out of 46) obtained in the first half of fermentation, and cluster VIII contained isolates obtained from the second half and end of fermentation.

3.4. Phenotypic characteristics related to probiotic potential

The results obtained from the sedimentation experiments showed that lactobacilli isolates had a strong auto-aggregating phenotype. This phenotype exhibited a normal distribution, with the largest number of isolates being included in the interval between 50% and 80% (Figure 3a). Four isolates, GG1ST0-4, HM2T0-63, GM2FT1-129 and HG2T1-138, showed the highest auto-aggregation values (more than 90%), while GM2FT4-288 showed values below 10%. On the contrary, the hydrophobicity distribution pattern was strongly skewed and showed that most of the isolates had values between 0% and 5% (Figure 3b). This could indicate that most of the lactobacilli were weakly hydrophobic, although some of them showed levels higher than 25% (GM2FT2-206, HM2T2-222, GG1FT3-230, GM2ST3-245, GM2FT3-246, HM2T4-308, HG1T5-330, and HG2T4-297).

Regarding bacteriocin production, the first screening indicated that some strains were able to inhibit the growth of the indicator pathogen microorganisms in the diffusion agar assays. However, a second assay with proteinase K revealed that the inhibition was in fact due to the production of lactic acid (data not shown). Therefore, none of the lactobacilli isolates had the capacity to produce bacteriocins under the assayed conditions. The results of the haemolytic and BSH tests also revealed the absence of these activities in all isolates.

Most of the isolates showed a low survival rate (%) after the SGD, with a high number of lactobacilli included in an interval of survival rate between 0.00% and 0.05%. However, a

few isolates showed high survival rates among those tested: 0.250% for GG3F-T3-238; 0.150% for GG1S-T3-229; 0.102% for GG1S-T1-84; 0.092% for JOLCA A1-71; 0.075% for GG2S-T1-91; 0.076% for GM2S-T1-127; and 0.055% for HG1T4-291). All of them showed greater survival rates than the reference probiotic strains used for comparison: 0.004% for *L. casei* Shirota; and 0.043% for *L. rhamnosus* GG. A group of strains showed survival rates close to 0.05%: 0.048% for GG2F-T1-96; 0.045% for GG2S-T2-168; 0.041% for GM1S-PRE-41; and 0.040% for HM2-T3-256.

After SGD, all isolates were sequentially subjected to the simulated pancreatic digestion. In general, a higher survival rate to the pancreatic than to the gastric digestion was noticed. The histogram corresponding to the partial survival in the simulated pancreatic digestion was clearly skewed (tail) to the right (data not shown), indicating the presence of some strains with relatively high survival rates. The partial survival rate for the pancreatic digestion was generally below 25%, although there were some strains particularly resistant, e.g., GGAS-T1-111 showing 56.7%, GG2F-T0-13 at 39.4%, GM2S-T0-36 at 36.7%), and finally GM2F-T5-327 at 36.1%. In comparison with the survival rate of the reference probiotic strains, 2.00% for *L. casei S*hirota and 2.14% for *L. rhamnosus* GG), the values found in this work were higher.

The overall lactobacilli survival in the digestive process with respect to the initial population is shown in Figure 4. The histogram was strongly skewed (tail) to the right. The overall rates were, in general, low and showed that most of the strains had percentages below 0.001%. There were only two strains with survival values between 0.007% and 0.009% while a few more were between 0.002% and 0.004%. The overall survival proportions of the probiotic control species were even lower.

3.5. Multivariate analysis of phenotypic characteristics related to probiotic potential

When subjecting the auto-aggregation, hydrophobicity, gastric digestion survival, pancreatic digestion survival, and overall digestion variables to PCA, using the isolates as grouping variables, only three eigenvalues higher than 1 were obtained. The study of the contribution of variables to factors (Table 2) showed that Factor 1 (which explained 29.54% variance) was mainly related to pancreatic digestion survival and overall survival, Factor 2 (21.61%) to auto-aggregation and gastric digestion survival, and Factor 3 (20.54%) to hydrophobicity. A projection of the variables onto the plane formed by the first two Factors (Figure 5a) clearly depicted this relationship but, in addition, also disclosed the link between pancreatic digestion and overall digestion as well as that between auto-aggregation and gastric digestion survival.

Figure 5b shows the projection of the isolates (cases) onto the plane formed by the two first Factors. There were a cloud of isolates around the center of the new axes (their identities have been omitted to improve readability); all of them can be considered to be very close to each other. However, there were also a few isolates separated from the rest. GG2S-T2-168 and GMSF-T5-327 were clearly distinguished. Their position was related to the negative side of Factor 1 and the average value for Factor 2; as a result, they are characterized by high values of pancreatic digestion survival and overall digestion (due to their negative relationship with Factor 1). PCA also allowed for the segregation of another group characterized by high values of auto-aggregation and gastric digestion (GM1F-T2-195 and GG3F-T3-238) and an average pancreatic and overall digestion survival. There were also other isolates with a slight segregation from the cloud of points (such as HM2-T2-222, GM2S-T0-36, GG4S-T1-111, GG2F-T0-13, and GG3F-T3-237) but discrimination criteria were more difficult to establish in this case. Table 3 shows the *L. pentosus* isolates with the most promising potential probiotic characteristics based on PCA analysis. The highest overall survival rate was observed in isolate GG2S-T2-168 (0.00804%, with a final population of ~3 $\log_{10}$ CFU ml^{-1} at

the end of digestive and pancreatic digestions) which also had the highest proportions of the auto-aggregation phenotype (75.61%) and hydrophobicity (8.52%). Isolate GM2FT5-327 had outstanding survival (0.00738%, with a final population of ~4.23 $\log_{10}$ CFU ml^{-1}), but its auto-aggregation and hydrophobicity values were lower than those of GG2S-T2-168. Isolates GG3F-T3-238 had survival rates comparable to those of the reference while isolates GM1F-T2-195 did not survive at all; however, both showed high auto-aggregation and hydrophobicity values.

4. Discussion

LAB starter cultures are widely used in combination with probiotic bacteria to produce fermented dairy derivatives (Vinderola, Bailo, & Reinheimer, 2003). However, there are an increasing number of consumers who are allergic to milk, or simply, prefer more variation with respect to probiotic sources. Products traditionally subjected to lactic acid fermentation during their processing are good candidates. Among others, table olives, the most common fermented vegetable product in western countries, are especially relevant due to their worldwide production, distribution, and progressively increasing consumption (Garrido-Fernández et al. 1997). Furthermore, table olives are also associated with the Mediterranean diet and enjoy a healthy and natural reputation. Thus, it is highly probable that presumptive probiotic table olives could be well accepted by consumers. In fact, probiotic table olives have already been proposed; however, the bacteria used for the fermentation process were isolated from humans (Lavermicocca et al. 2005a, 2005b; Valerio et al. 2006; De Bellis et al. 2010). In this work, we have selected lactobacilli isolates from different spontaneous industrial green table olive fermentations with promising *in vitro* phenotypic characteristic related to their probiotic potential. The fermentations from which they were selected belonged to typical Spanish and directly brined green olive processes and the strains could *a priori* have a wide

application spectrum. The probiotic products produced with them will thus have the invaluable advantage of their origin without the introduction of any strange factor to their processing.

In traditional olive fermentations, the LAB species mentioned were numerous (Garrido Fernández et al. 1997), but in recent studies the species isolated have been more limited. *L. plantarum* (Mourad & Nour-Eddine, 2006) was predominant in naturally fermented Algerian green olives, while *L. plantarum* and *L. pentosus* were identified in Gallega fermentations (Oliveira et al. 2004). *L. casei* and *L. plantarum* were detected in naturally fermented Sicilian olives (Randazzo, Restuccia, Romano, & Caggia, 2004) and *L. pentosus* and *L. paraplantarum* were isolated from Arbequina directly brined olives (Hurtado, Reguant, Esteve-Zarzoso, Bordons, & Rozès, 2008). However, recently, *L. pentosus* is gaining prominence (Panagou, Tassou, & Katsaboxakis, 2003; Segovia-Bravo, Arroyo López, García García, Durán Quintana, & Garrido Fernández, 2007a, 2007b; Panagou, Schillinger, Franz, & Nychas, 2008; Peres, Catulo, Brito, & Pintado, 2008). The results of this work are in agreement with this trend, and most of the isolated strains belonged to this last species, with a reduced lactobacilli inter-specific biodiversity although the strains originated from different factories and elaboration processes. On the contrary, an intra-specific diversity was found within the *L. pentosus* species, and the isolates characterized in this study formed 9 different clusters, although two main groups (VI and VII) accounted for the majority of the microorganisms. In addition, no definitive differentiation could be observed based on the processing plant of origin, olive cultivar or day of isolation (Figure 2). These results prevent any speculation regarding a plant-specific population or strain evolution during the fermentation process.

Selection criteria for LAB to be used as functional starter cultures have been recently updated (Ammor & Mayo, 2007). In this work, those phenotypic characteristics considered relevant for a first criterion of selection based in their probiotic potential were evaluated.

BSH activity was not found in any of the isolates. However, interpretation of this result is not straightforward because, although the ability of probiotic strains to hydrolyze bile salts is usually included among the criteria for probiotic strain selection, the BSH activity has also been mooted to be potentially detrimental to the human host and thus it is not yet completely clear that it could be a desirable trait in a probiotic bacterium.

Adhesion ability to the intestinal epithelium is another important criterion for selecting potentially probiotic strains (Ouwehand, Kirjavainen, Shortt, & Salminen, 1999). It has been shown that those strains able to adhere to cell monolayers also show auto-aggregation and hydrophobicity characteristics as determined by microbial adhesion to hydrocarbons. Therefore, these two traits may be used for preliminary screening to identify potentially adherent isolates (Del Re, Sgorbati, Miglioli, & Palenzona, 2000). However, results should be interpreted with caution because this adherence does not necessarily mean an *in vivo* adhesion. In our study, the auto-aggregation phenotype was fairly widespread and had almost a normal distribution among most of the strains showing values higher than 50%. However, hydrophobicity had a strongly skewed distribution, with some isolates showing values above 25%. Isolate HM2-T2-222 had a good auto-aggregation phenotype (nearly 58%) and an important resistance to gastric and pancreatic digestions.

Because of the unfavourable environmental conditions for microorganism survival in the stomach, where the pH is around 2.0 (Fernández, Boris, & Barbés, 2003), it is essential to select probiotic strains with high tolerance to acidic conditions. Similarly, it is important to select probiotic strains with high resistance to bile because they could develop better in the upper intestine (Gilliland, Staley, & Bush, 1984). In this study, survival during transit through

the gastro intestinal tract has been considered as one of the most critical factors because the survival rate is extremely low in the gastric digestion and only a reduced number of strains were able to pass the first step. The sequential gastrointestinal and pancreatic simulated digestions have been applied because the independent behavior of strains to each of them might lead to a good partial choice but inappropriate global selection. Overall survival should thus be more realistic than results obtained with the application of separate test for each type of digestion; results for some isolates of this work were higher than for currently considered probiotic strains used in dairy products (reference strains).

The PCA was useful to show the relationships among variables themselves and with the new Factors. Projection of the cases onto the two first Factors clearly differentiated strains GG2S-T2-168 and GM2F-T5-327, in agreement with the overall survival rate criterion. Other strains (GM1F-T2-195 and GG3F-T3-238) also far apart from the rest, could have, in principle, limited application as potential probiotics because their differences were mainly linked to characteristics related to high auto-aggregation and gastric digestion survival.

4. Conclusion

Certain *L. pentosus* isolates showed promising probiotic and differentiated characteristics as was proven by PCA. GG2S-T2-168 showed not only high survival rates but also high values for auto-aggregation and hydrophobicity. The GM2F-T5-327 isolate had a moderate auto-aggregation value although with a low level of hydrophobicity but similar to that of the reference probiotic strains used as the control. However, final selection would also depend on their real *in vivo* probiotic properties as well as on other technological characteristics like survival in brine, production of lactic acid during fermentation or adhesion to olive surface, among others.

Acknowledgements

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Figure legends

Figure 1. Changes in lactic acid bacteria counts vs. time (at selected interval levels), according to processing system and cultivar. GGV, Spanish style green Gordal olives; GMV, Spanish style green Manzanilla olives; HJG, directly brined Gordal olives; HJM, directly brined Manzanilla olives; AMC, directly brined Aloreña olives stored in cold room; AMP, directly brined Aloreña olives stored in covered yard at ambient temperature. Vertical bars denote 95% confidence intervals.

Figure 2. Dendrogram generated after cluster analysis of the digitized rep-PCR fingerprints of lactobacilli. An 80% coefficient of similarity was arbitrarily selected to identify the clusters, which are indicated with roman numerals.

Figure 3. Histograms showing the distribution of the auto-aggregation (a) and hydrophobicity (b) characters in lactobacilli isolates.

Figure 4. Histogram of the overall simulated gastric and pancreatic digestion survival, expressed as percentage of the initial population.

Figure 5. Projection of the variables (a) and cases/isolates (b) onto the plane formed by the two first factors deduced from PCA.

Table 1. Physicochemical conditions of the brines obtained from the different table olive elaborations studied in this work. Standard errors in parentheses.

		NaCl (g l^{-1})		Combined acidity (mEq l^{-1})		pH		Titratratable acidity (g l^{-1})	
Elaboration	Cultivar	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Directly brined green olives	Aloreña	91.0 (±3.8)	69.5 (±3.8)	25 (±2)	72(±2)	4.67 (±0.06)	4.31 (±0.06)	1.1(±0.3)	5.9(±0.3)
	Gordal	69.3 (±1.2)	49.5 (±1.2)	15 (±2)	48(±2)	4.45 (±0.08)	3.91 (±0.07)	1.3 (±0.3)	5.6(±0.3)
	Manzanilla	59.5 (±1.4)	45.2 (±1.4)	29(±2)	75(±2)	4.25 (±0.15)	4.00 (±0.03)	2.7(±0.3)	7.7(0.3)
Spanish style green olives	Gordal	65.5 (±1.5)	64.5 (±1.5)	105(±6)	126(±4)	5.55 (±0.12)	3.84 (±0.09)	1.5(±0.6)	11.3(0.6)
	Manzanilla	64.1 (±0.7)	63.2 (±0.7)	117(±1)	122(±1)	5.70 (±0.48)	3.89 (±0.05)	1.5(±0.1)	11.6(±0.1)

Table 2. Contribution of variables to the factors in the PCA based on correlations.

Variable	Factor 1	Factor 2	Factor 3
Auto-aggregation	0.0662	0.3051	0.0190
Hydrophobicity	0.0147	0.0218	0.8667
Gastric digestion survival	0.0040	0.6076	0.0817
Pancreatic digestión survival	0.4706	0.0655	0.0011
Overall digestión	0.4445	<0.0001	0.0315

Table 3. *In vitro* phenotypic characteristics of the selected lactobacilli isolates selected by PCA.

Isolate reference	Species	Overall survival rate %	Residual population* $CFUml^{-1}$	Auto-aggregation (%)	Hydrophobicity (%)
GG2S-T2-168	*L. pentosus*	0.00804	3.0	76.51	8.52
GM2F-T5-327	*L. pentosus*	0.00738	4.2	27.00	1.30
GM1F-T2-195	*L. pentosus*	0.00000	no survival	51.23	5.15
GG3F-T3-238	*L. pentosus*	0.00007	2.8	63.05	1.55
Control	*L. casei* *S*hirota	0.00007	3.2	33.39	1.13
Control	*L. rhamnosus* GG	0.00091	2.9	39.06	0.23

Note: *overall absolute survival counts after gastric and pancreatic digestions.

Figure 1

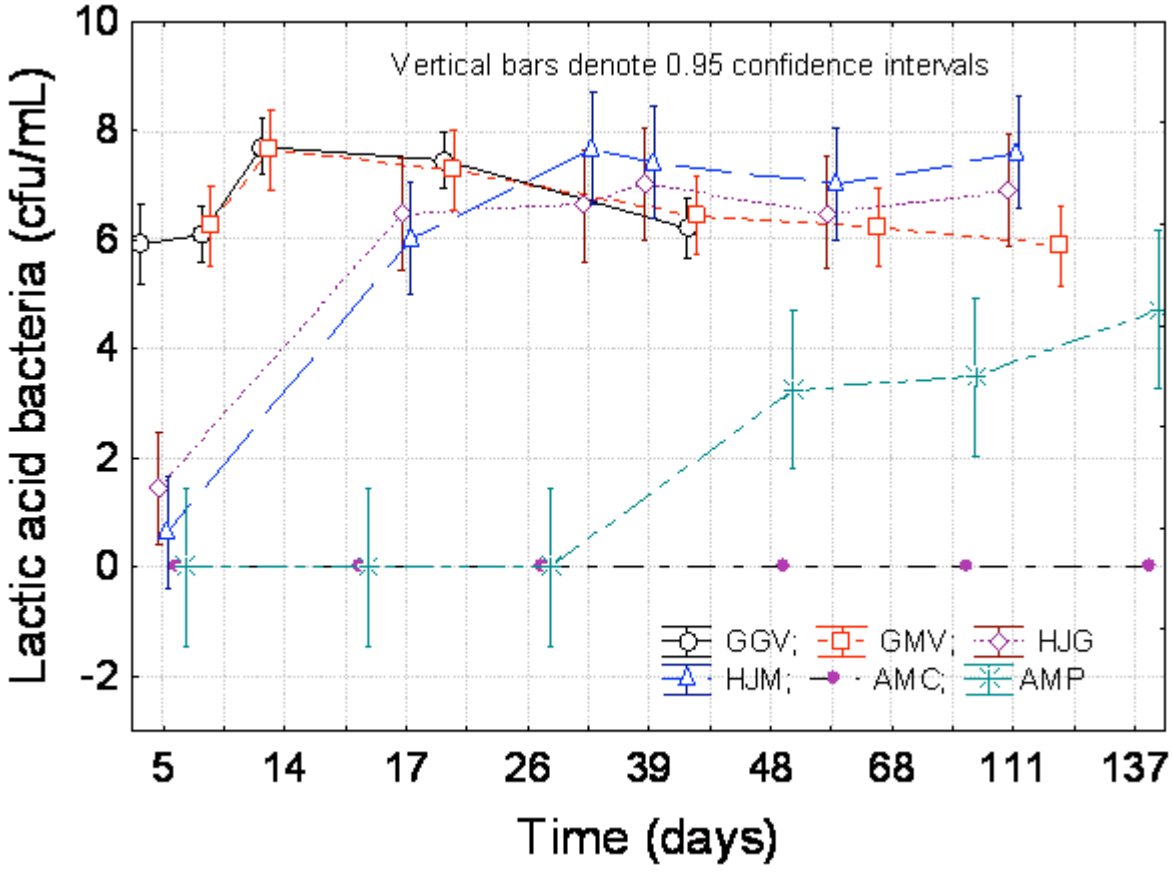
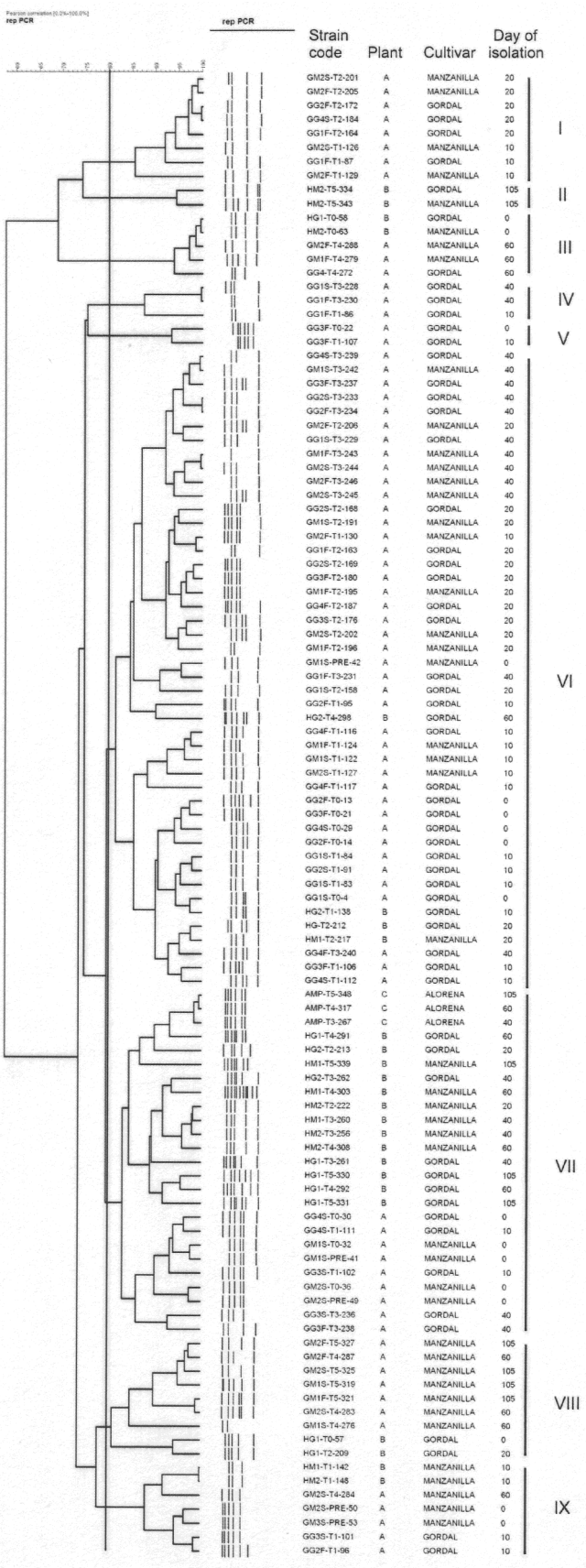

Vertical bars denote 0.95 confidence intervals
Lactic acid bacteria (cfu/mL)
10
8
6
4
2
0
-2
5
14
17
26
39
48
68
111
137
Time (days)
GGV;
GMV;
HJG
HJM;
AMC;
AMP

Figure 2


rep PCR
rep PCR
Strain code
Plant
Cultivar
Day of isolation
GM2S-T2-201 A MANZANILLA 20
GM2F-T2-205 A MANZANILLA 20
GG2F-T2-172 A GORDAL 20
GG4S-T2-184 A GORDAL 20
GG1F-T2-164 A GORDAL 20
GM2S-T1-126 A MANZANILLA 10
GG1F-T1-87 A GORDAL 10
GM2F-T1-129 A MANZANILLA 10
HM2-T5-334 B GORDAL 105
HM2-T5-343 B MANZANILLA 105
HG1-T0-58 B GORDAL 0
HM2-T0-63 B MANZANILLA 0
GM2F-T4-288 A MANZANILLA 60
GM1F-T4-279 A MANZANILLA 60
GG4-T4-272 A GORDAL 60
GG1S-T3-228 A GORDAL 40
GG1F-T3-230 A GORDAL 40
GG1F-T1-86 A GORDAL 10
GG3F-T0-22 A GORDAL 0
GG3F-T1-107 A GORDAL 10
GG4S-T3-239 A GORDAL 40
GM1S-T3-242 A MANZANILLA 40
GG3F-T3-237 A GORDAL 40
GG2S-T3-233 A GORDAL 40
GG2F-T3-234 A GORDAL 40
GM2F-T2-206 A MANZANILLA 20
GG1S-T3-229 A GORDAL 40
GM1F-T3-243 A MANZANILLA 40
GM2S-T3-244 A MANZANILLA 40
GM2F-T3-246 A MANZANILLA 40
GM2S-T3-245 A MANZANILLA 40
GG2S-T2-168 A GORDAL 20
GM1S-T2-191 A MANZANILLA 20
GM2F-T1-130 A MANZANILLA 10
GG1F-T2-163 A GORDAL 20
GG2S-T2-169 A GORDAL 20
GG3F-T2-180 A GORDAL 20
GM1F-T2-195 A MANZANILLA 20
GG4F-T2-187 A GORDAL 20
GG3S-T2-176 A GORDAL 20
GM2S-T2-202 A MANZANILLA 20
GM1F-T2-196 A MANZANILLA 20
GM1S-PRE-42 A MANZANILLA 0
GG1F-T3-231 A GORDAL 40
GG1S-T2-158 A GORDAL 20
GG2F-T1-95 A GORDAL 10
HG2-T4-298 B GORDAL 60
GG4F-T1-116 A GORDAL 10
GM1F-T1-124 A MANZANILLA 10
GM1S-T1-122 A MANZANILLA 10
GM2S-T1-127 A MANZANILLA 10
GG4F-T1-117 A GORDAL 10
GG2F-T0-13 A GORDAL 0
GG3F-T0-21 A GORDAL 0
GG4S-T0-29 A GORDAL 0
GG2F-T0-14 A GORDAL 0
GG1S-T1-84 A GORDAL 10
GG2S-T1-91 A GORDAL 10
GG1S-T1-83 A GORDAL 10
GG1S-T0-4 A GORDAL 0
HG2-T1-138 B GORDAL 10
HG-T2-212 B GORDAL 20
HM1-T2-217 B MANZANILLA 20
GG4F-T3-240 A GORDAL 40
GG3F-T1-106 A GORDAL 10
GG4S-T1-112 A GORDAL 10
AMP-T5-348 C ALORENA 105
AMP-T4-317 C ALORENA 60
AMP-T3-267 C ALORENA 40
HG1-T4-291 B GORDAL 60
HG2-T2-213 B GORDAL 20
HM1-T5-339 B MANZANILLA 105
HG2-T3-262 B GORDAL 40
HM1-T4-303 B MANZANILLA 60
HM2-T2-222 B MANZANILLA 20
HM1-T3-260 B MANZANILLA 40
HM2-T3-256 B MANZANILLA 40
HM2-T4-308 B MANZANILLA 60
HG1-T3-261 B GORDAL 40
HG1-T5-330 B GORDAL 105
HG1-T4-292 B GORDAL 60
HG1-T5-331 B GORDAL 105
GG4S-T0-30 A GORDAL 0
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GM1S-T0-32 A MANZANILLA 0
GM1S-PRE-41 A MANZANILLA 0
GG3S-T1-102 A GORDAL 10
GM2S-T0-36 A MANZANILLA 0
GM2S-PRE-49 A MANZANILLA 0
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GG3F-T3-238 A GORDAL 40
GM2F-T5-327 A MANZANILLA 105
GM2F-T4-287 A MANZANILLA 60
GM2S-T5-325 A MANZANILLA 105
GM1S-T5-319 A MANZANILLA 105
GM1F-T5-321 A MANZANILLA 105
GM2S-T4-283 A MANZANILLA 60
GM1S-T4-276 A MANZANILLA 60
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HM1-T1-142 B MANZANILLA 10
HM2-T1-148 B MANZANILLA 10
GM2S-T4-284 A MANZANILLA 60
GM2S-PRE-50 A MANZANILLA 0
GM3S-PRE-53 A MANZANILLA 0
GG3S-T1-101 A GORDAL 10
GG2F-T1-96 A GORDAL 10
I
II
III
IV
V
VI
VII
VIII
IX

Figure 3

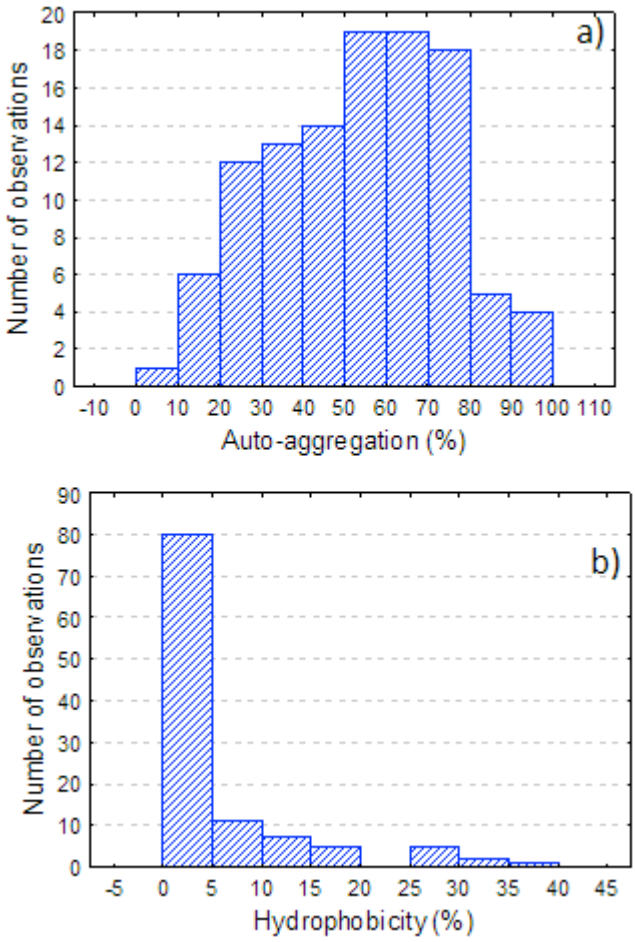

a)
Number of observations
Auto-aggregation (%)
b)
Number of observations
Hydrophobicity (%)

Figure 4

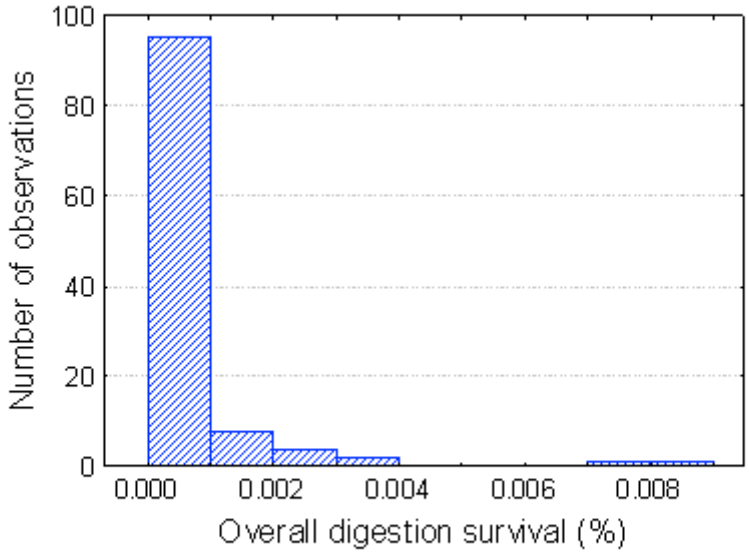
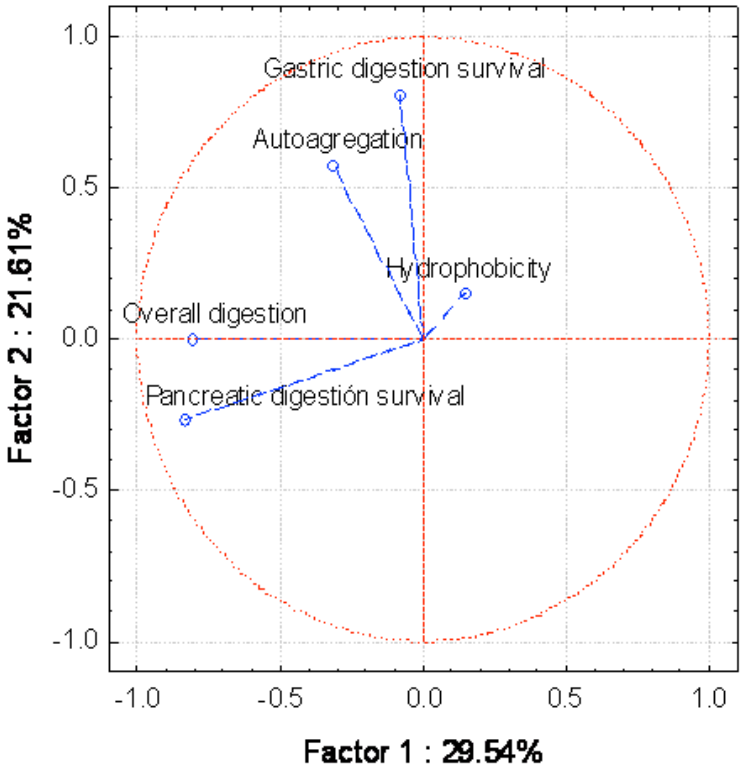
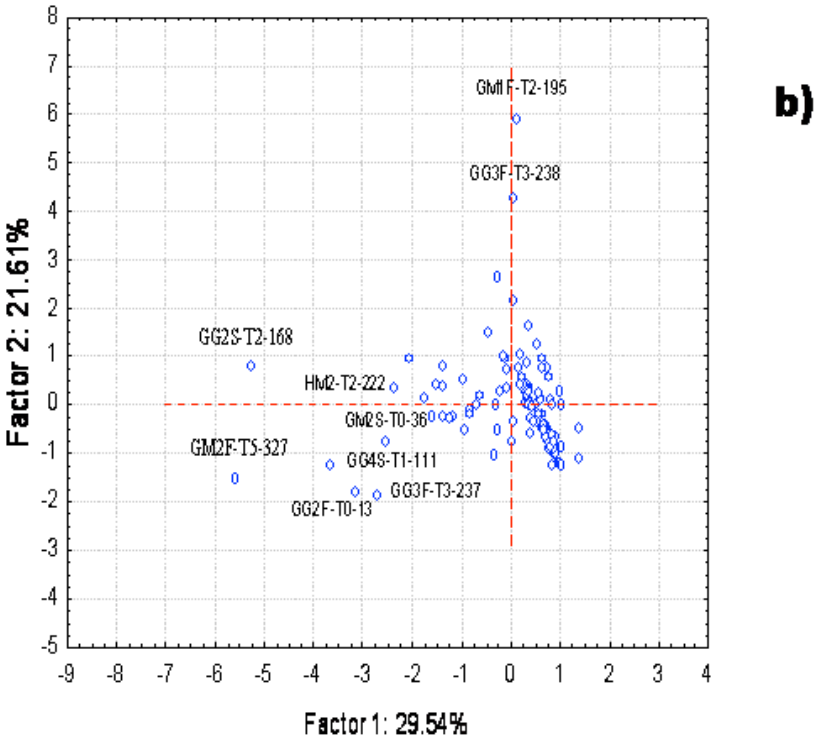

100
80
60
40
20
0
Number of observations
0.000
0.002
0.004
0.006
0.008
Overall digestion survival (%)

Figure 5


a)
Gastric digestion survival
Autoagregation
Hydrophobicity
Overall digestion
Pancreatic digestión survival
Factor 2 : 21.61%
Factor 1 : 29.54%
1.0
0.5
0.0
-0.5
-1.0
-1.0
-0.5
0.0
0.5
1.0


b)
GM1F-T2-195
GG3F-T3-238
GG2S-T2-168
HM2-T2-222
GM2S-T0-36
GM2F-T5-327
GG4S-T1-111
GG3F-T3-237
GG2F-T0-13
Factor 2: 21.61%
Factor 1: 29.54%