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**Identification, stress tolerance and antioxidant activity of lactic acid bacteria isolated from
tropically-grown fruits and leaves**

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ABSTRACT

Out of six samples of tropically grown fruits and leaves, ten lactic acid bacteria belonging *Leuconostoc*, *Weissella* and *Lactobacillus* species were isolated and identified by 16S gene sequencing and (GTG)₅ fingerprinting. Acidification kinetics determined from BHI broth cultures showed genus-related patterns. In particular, *Weissella cibaria* appeared to act as a potent acidifier. Tolerance to acid, oxidative or salt stress of isolates was highly variable and strain dependent. Isolate S14 (*Leuconostoc pseudomesenteroides*) growth was not affected by the presence of 0.05% H₂O₂ while *Lactobacillus* spp. isolates (S17 and S29) were the most tolerant to pH 4.5. The growth of four isolates, S5 (*Leuconostoc mesenteroides*), S14 and S10 (*Leuc. pseudomesenteroides*) and S27 (*W. cibaria*) was not affected by 5% NaCl. Nutritional beneficial properties were examined through measurement of antioxidant activities of short-term fermented pineapple juice, such as LDL oxidation and polyphenol content, and through exopolysaccharide formation from sucrose. Two isolates, S14 and S27 increased antioxidant capacity of pineapple juice. The robust capacity of *W. cibaria* and of *Leuc. pseudomesenteroides* for vegetable lactic fermentation aimed to ameliorate food nutritional and functional quality was highlighted.

Keywords: diversity, *Weissella* spp., *Leuconostoc* spp., antioxidant, stress adaptation

INTRODUCTION

Lactic acid bacteria (LAB) cover many different species, with very diverse physiological traits and ecology. They can be used as starters, probiotics, for ingredient biosynthesis or for bio-preservation. Lactic acid fermentation of fruits and vegetables has regained attention over the last decade due to the multiple health advantages related to the consumption of fruits and vegetables compared to animal products and to the fast growing demand of healthy foods by increasingly conscious consumers. When applied to table olives, cabbage for sauerkraut or kimchi production or small fruits for pickles, this simple and valuable biotechnology results in a safety increase, in prolonged shelf-life and in changes of nutritional and sensorial properties (Bourdichon et al. 2012; Hugenholtz 2013; Juodeikiene et al. 2012; Rodriguez et al. 2009). In particular, the effects of lactic acid fermentation on antioxidant activity of fruits and vegetables were investigated (Di Cagno et al. 2011, 2009; Filannino et al. 2013; Noumo et al. 2013; Othman et al. 2009; Park et al. 2011; Sun et al. 2009; Wu et al. 2011). Depending on the studies, a more or less marked decrease or increase in antioxidant activity and changes in phenolic compounds have been reported. The differential effects of lactic acid fermentation observed between studies can be explained by the diversity of substrates, fermentation protocols and analytical methods used in these studies, but also by the diversity of LAB. Edible fruits and leaves in warm-climate countries are often traditionally preserved as lactic fermented foods (Franz et al. 2014; Lan et al. 2013; Ng et al. 2011; Nguyen et al. 2013). In these cases, home-made products result from spontaneous fermentation or back-slopping (Lan et al. 2013; Liu et al. 2011; Oguntoyinbo et al. 2007) and fermentative bacteria originate from fruit phyllosphere and kitchen equipment. Spontaneous fermentation results from a competition between microorganisms where the most adapted ones will prevail. Hence, food fermentation processes are associated with several stresses, depending on the growth medium composition and other environmental conditions (Giraffa 2004; Serrazanetti et al. 2013). For these reasons, autochthonous bacteria, well-adapted to their ecological niche, are recognized as the most efficient to perform fermentation.

In this study, we first aimed to assess the biotechnological diversity of LAB isolates from the surface of papaya, tomato or sliced cabbage grown under tropical warm-climate. A second objective was to select LAB for their future use as starter for healthy fermented fruit or vegetable juices. The Victoria cultivar of pineapple, produced in South West areas of Indian Ocean, recognized for its sensorial properties, was chosen as fermentation substrate. As bacteria ability to resist to acid juice and to maintain or increase antioxidant activity level is critical for fruit or vegetable fermentation, relevant phenotypic traits as growth yield, acidification rate, stress tolerance, antioxidant activity and exopolysaccharide formation were determined.

MATERIALS AND METHODS

Biological materials

LAB were isolated from tomatoes (*Lycopersicon esculantum*), sliced white cabbage (*Brassica oleacera* var. *capitata*) and papaya (*Carica papaya*) grown in Reunion Island and isolates were stored at -80°C.

These food materials were obtained from local markets and stored for 24 h at 4°C in sterile bags before analysis. Sliced cabbage was directly used, whereas tomato and papaya were cut: a 1 cm-thick slice in the median part of the fruit was cut with a sterile scalpel and 25 g were sampled from the center of the slice to the external arc of circle. For isolation, 25 g sample was mixed with 25 g buffered peptone water in a stomacher for 1 min, and then serial decimal dilutions in the same buffer were spread on selective agar. All isolates share the ability to grow on MRS agar with cycloheximide and are catalase negative. *Lactobacillus plantarum* DSM2601 was used as reference strains for all experiments.

A single batch of commercial pasteurized pineapple juice (Pur Jus Ananas Victoria de la Réunion, ALBIUS) was used for all experiments.

Culture conditions

Isolates and DSM2601 were grown on MRS agar at 37°C for 72 h.

For isolate reactivation, one or two colonies or a loop of -80°C stock were suspended in 9 mL of BHI (Brain Heart Infusion) and incubated at 37°C for 48 h. Afterwards cultures were homogenized by vortex and 0.2 mL of the medium was used to inoculate 9 mL of BHI. This pre-culture was incubated at 37°C for 72 h. After 72 h, another tube of BHI broth was inoculated from this pre-culture and incubated at 37°C for 24 h. In BHI, cell population was estimated by measuring absorbance at 660 nm wavelength.

An adequate volume of juice was poured in autoclaved containers. The inoculation volume corresponded to an initial OD at 660 nm of 0.05. For short-term cultures, a volume of 40 mL of inoculated juice was incubated in 250 mL Erlenmeyer flasks at 25°C in a shaking incubator at 150 rpm for 48 h. Pasteurized juice was used as control. For each isolate and for both controls, the experiment was performed three times from independent pre-cultures.

Identification

DNA extraction was performed using Instagen protocol (Instagen Matrix, Biorad). The supernatant containing DNA was stored at -20°C.

Two primers (Eurogentec), RD1m 5'-GGM-TAC-CTT-ACG-AYT-TC-3' and FD1m 5'-AGA-GTT-TGA-TCH-TGG-CTC-AG-3', were used for DNA amplification. The reaction mixture contained buffer 1x, dNTP 2 mM, each primer 0.3 µM, MgCl₂ 2.5 mM, and *Taq* polymerase 0.02 U.µL⁻¹ (Diamond, Eurogentec).

The PCR reaction was performed in a 45 µL-volume with 10 µL of DNA solution. The PCR program comprised 35 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 55°C and elongation for 1 min at 72°C. The cycles were preceded by a denaturation step at 94°C for 3 min and followed by an elongation step at 72°C for 10 min. PCR was performed with a Bio-Rad S100 Thermal Cycler. PCR products were separated by electrophoresis (60 min at 100 V) on 0.8% agarose gel in TAE 0.5x. DNA was detected with a GelDoc UV-illuminator system (Bio-Rad) after staining with ethidium bromide. The 1 kb DNA ladder (Invitrogen) was used.

PCR product purification was performed with GenElute PCR Clean-up Kit (Sigma-Aldrich). PCR product was sequenced by Sanger method with FD1m primer (Eurofins). The obtained sequence was

compared to Ribosomal Database Project (Cole et al. 2014) and NCBI Nucleotide with BLASTN program. Alignments were performed with CLUSTAL OMEGA program (EMBL) (Sievers et al. 2011) and trees were built up with JalView (Waterhouse et al. 2009).

A rep-PCR method based on (GTG)₅ primer was used to differentiate species. Reference strains DSM2601 (*Lb. plantarum*), DSM 20174 (*Lb. plantarum* Type strain), DSM 20188 (*Leuconostoc citreum*), DSM 5625 (*Leuconostoc pseudomesenteroides*), DSM 20193 (*Leuc. pseudomesenteroides* Type strain), DSM 15830 (*Weissella koreensis* Type strain), DSM 20196 (*Weissella confusa* Type strain), DSM 5625 (*Weissella cibaria*), DSM 15878 (*W. cibaria* Type strain) were used as control.

PCR was performed according to Versalovic et al. (1994) with some modifications. The PCR reaction was performed in 50µL containing 10µL of DNA solution. The reaction mixture contains 1X buffer, dNTP 2mM, (GTG)₅ primer 5'-GTG-GTG-GTG-GTG-GTG-3' 2µM (Eurogentec), MgCl₂ 4mM and *Taq* polymerase 0.0375 U.µL⁻¹ (5 PRIME). The (GTG)₅ PCR program comprised 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 40°C and elongation for 3 min at 72°C. The cycles were preceded by a denaturation step at 94°C for 5 min and followed by an elongation step at 72°C for 10 min. PCR was performed with a Bio-Rad S100 Thermal Cycler. PCR products were separated by electrophoresis in a 0.8% agarose gel (20 X 20) for 6 hours at 80 V in 1x TAE. DNA was detected as previously described by comparison with 1 kb and 100 bp DNA ladders (Nippon Genetics).

Electrophoretic profiles were analyzed with Phoretix 1D Pro software (TotalLab, UK). Nearest Neighbor algorithm and Pearson coefficient correlation were used to build the dendrogram.

Exopolysaccharide (EPS) production

Screening for EPS producing phenotype was carried out from colony aspect after growth at 30°C during 72 h on MRS medium supplemented with 40 g.L⁻¹ of sucrose. The phenotype was rated according to mucoid appearance with attributes: (-) non mucoid/non EPS-producing; (+) EPS-producing; (++) high EPS producing and (+++) very high EPS producing. The colony aspect of EPS producing isolates was described as liquid or creamy.

Acidification kinetics

Bacterial isolates were reactivated for 48 h at 37°C in BHI broth. They were inoculated in 150 mL of BHI broth. Volume of inoculation was calculated corresponding to an initial OD at 660 nm of 0.05. Cultures were incubated at 25°C in a water bath under agitation at 100 rpm with a magnetic stirrer. A pH probe connected to an automated acquisition system (Consor multi-parameter analyser C3060) was plunged in each culture. The medium pH values were acquired for 72 h with a time interval of 5 min with CINAC Software. Acidification curve was obtained by plotting pH as a function of time. Each culture was performed in duplicate. Lag time λ was the time corresponding to an initial pH variation below 0.1. Minimal pH pH_{min} was the final constant pH value. Maximum acidification rate V_M , time to reach the maximum acidification rate without the lag phase t_M and pH at maximal acidification rate pH_M were determined from $dpH/dt = f(t)$ curves obtained with Regressi Software.

Adaptation

Bacterial isolates were grown for 48 h at 37°C in BHI broth pH 6.8 and 0.2 mL fraction of this bacterial suspension was inoculated into 9-mL assay broths. The control medium used was BHI broth pH 6.8. For acid stress adaptation, BHI broth acidified to pH 4.5 with HCl 2M was used. For osmotic stress adaptation, BHI broth containing 5% NaCl was used. For oxidative stress, BHI broth containing 0.025 % or 0.05 % H_2O_2 or 0.07 % H_2O_2 was used. OD at 660 nm was measured just after inoculation (OD_0) and after incubation at 37°C for 48 h (OD_{48}). Results were expressed as $\text{Log}(OD_{48}/OD_0)$.

All adaptation assays were performed in triplicate for each isolate.

Analysis of pineapple juice

All chemicals were purchased from Sigma Aldrich. Reducing sugar content was determined according to Miller method (Miller 1959). Cell counts were performed after inoculation and after 48 h of incubation by MRS plating. Seven different assays were used to evaluate the antioxidant activity. Each test was performed in independent triplicates for each sample and control.

Oxygen Radical Absorbance Capacity (ORAC) analysis

Oxygen Radical Absorbance Capacity (ORAC) analysis was used to determine antioxidant capacity of 5000-fold diluted, fermented or non-fermented, pineapple juices. The antioxidant activity of the

sample was determined from its capacity to reduce fluorescein oxidation by peroxy radicals generated with AAPH (2,2'-Azobis [2-methyl- propionamidin]dihydrochloride). Briefly, in a black 96-well plate, 25 µL of sample were mixed with 150 µL of 0.0838 µM fluorescein prepared in 1 M phosphate buffer pH 7.4. After 15 min of incubation at 37°C in the TECAN INFINITE M200 spectrofluorimeter, 25 µL of 153 mM AAPH were added in each well. Decrease in fluorescence corresponding to fluorescein oxidation was measured at excitation and emission wavelengths of 485 and 530 nm respectively. Trolox at concentrations of 20, 50 and 100 µM was used as standard. The area under the curve (AUC) was determined automatically for each sample and for each Trolox concentration. Standard curve was established by plotting AUC versus Trolox concentration. Antioxidant activity was expressed as µM Trolox equivalent.

Erythrocytes hemolysis

In this assay, antioxidant activity of a sample corresponds to its capacity to inhibit erythrocyte oxidation by radicals generated from AAPH. Erythrocytes were separated from the plasma by centrifugation at 1000 x g for 5 min. The supernatant was removed and the same volume of 0.15 M NaCl solution was added. This washing step was repeated three times. Afterwards, erythrocyte solution was reconstituted by adding a volume of NaCl solution equivalent to initial plasma volume. In a 96-well plate, 100 µL of 50-fold diluted erythrocytes were mixed with 10,000-fold diluted samples in 0.15 M NaCl. Then, 40 µL of 0.5 M AAPH solution was added in each well. The microplate was incubated at 37°C and hemolysis was followed by the decrease of absorbance at 450 nm (FLUOSTAR – BMG France). Omega-5 software was used to acquire absorbance values. Results are expressed in half time of hemolysis (HT50), determined from GraphPad Prism 2.01 with a Boltzman sigmoidal nonlinear regression. The equation was: $Y = Abs_i + \frac{Abs_f - Abs_i}{1 + \exp\left(\frac{V_{50} - x}{slope}\right)}$ where Y is the absorbance at 450 nm, Abs_i the initial absorbance at 450 nm, Abs_f the final absorbance at 450 nm, and V-50 the half time hemolysis, expressed in min, and slope is the steepness of the curve.

OD 280nm measurement

Pineapple juice fermented or not was diluted 10-fold in ultrapure water, before the measurement of OD at 280 nm. Gallic acid was used as standard. Results are expressed as g.L⁻¹ gallic acid equivalent (GAE).

Determination of total phenolic content

The Folin-Ciocalteu method with gallic acid (GA) as standard was used and samples were 100-fold diluted. In a 96-well plate, 15 µL of Folin reagent was mixed with 30 µL of each sample or standard. After incubation for 4 min at ambient temperature, 60µL of Na₂CO₃ 700 mM was added to stop the reaction. Then 195 µL of distilled water was added. After incubation for 1 h at room temperature, absorbance at 760 nm was measured. Results were expressed as g.L⁻¹ gallic acid equivalent (GAE).

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging activity

ABTS is oxidized by potassium persulfate K₂S₂O₈ to generate cation radical ABTS^{o+} which absorbs at 734 nm. Antioxidant compounds degrade this radical, resulting in a decrease of absorbance at 734 nm. To generate the cation radical ABTS^{o+}, 5 mL of a solution of 7 mM ABTS was added to 5 mL of 2.45 mM potassium persulfate. ABTS^{o+} solution was then diluted in methanol in order to obtain an absorbance of 0.7 at 734 nm. Samples were 5-fold diluted in ultrapure water. In a transparent 96-well plate, 280 µL of ABTS^{o+} was mixed with 20µL of sample. The blank was obtained with 20 µL of ultrapure water. After 5 min of incubation at 30°C in the Tecan Infinite M200 spectrofluorimeter, absorbance at 734 nm was measured. Trolox at concentrations 50 µM to 250 µM was used as standard. The % of inhibition was expressed for Trolox and for samples with the formula: % of

$$\text{inhibition} = 100 - 100 * \frac{\text{Abs sample}}{\text{Abs blank}}.$$

The curve indicating % of inhibition *versus* Trolox concentrations was used as standard curve.

Antioxidant activity was expressed in mM TE.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

Diphenyl-2-picrylhydrazyl (DPPH) assay was used to determine the free radical scavenging activity of fermented and non-fermented pineapple juice. In a 96-well plate, 150 µL of DPPH solution (0.2 mM in methanol) was mixed with 50 µL of 5-fold diluted sample. The mixture was then kept at room

temperature for 30 min in the dark. The absorbance of the mixture was measured at 517 nm using microplate reader (Fluostar). Results were expressed as a % of inhibition of sample versus control. Trolox was used as positive control.

Low-density lipoprotein (LDL) oxidation

LDL (1.019–1.055 g.mL⁻¹) were isolated by sequential ultracentrifugation of pooled plasma from normolipidemic subjects (Beckman centrifuge). After dialysis against PBS pH 7.4, LDL were assayed for protein content by the bicinchoninic acid method and stored at 4°C in the dark for no longer than 15 days.

LDL oxidation was determined by measuring conjugated diene formation. Oxidation of LDL was conducted with 50 µL of 1.96 µg.µL⁻¹ of LDL protein in the presence of 30 µL samples (10,000-fold dilution). Negative control consisted of 30 µL of distilled water. Reaction was started by adding 20 µL of CuSO₄ solution (80 µM). Conjugated diene absorbance was measured by spectrophotometry at 234 nm every 5 min during 2 h at 37°C by using a microplate reader (Fluostar). Results are expressed in half time oxidation (V-50), obtained from GraphPad Prism 2.01 with a Boltzman sigmoidal nonlinear regression, as for erythrocyte hemolysis assay. V-50 corresponds to the half time oxidation, expressed in min.

Statistics

All values are expressed as mean ± standard deviation.

Variance analysis and Student t-test have been used to compare the different treatments. If a significant difference was pointed out, Bonferroni test was used to test the difference between each treatment and control. Principal component analysis was performed with Pearson(n) matrix.

The statistical analysis was performed using GraphPad Prism 2.01 (GraphPad Software, Inc) or XLSTAT (Addinsoft). Degrees of significance are indicated as follow: * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

RESULTS

Isolation and molecular identification

LAB population was determined from the different samples of papaya, tomato and sliced cabbage, purchased from local markets (Table1). The surface of the two fruits was characterized by LAB population comprised between 5.1 and 5.8 \log_{10} cfu.g⁻¹, while sliced cabbage contained higher LAB population with 9.2 to 10.0 \log_{10} cfu.g⁻¹. Isolates were recovered from these samples on the basis of colony morphological differences and microscopy differences. Out of 64 LAB isolates, 10 isolates from six samples were chosen based on morphological differences and identified by 16S rRNA coding region partial sequencing (Table 1). They belong to three different genera. *Leuconostoc* spp. was the most frequent. Isolates from papaya covered the three genera.

In order to distinguish species with highly identical 16S rRNA genomic sequences, a rep-PCR was applied. Similar profiles were obtained for isolates S9 and S13, S17 and S29, S5 and S6 and S12 and S27 respectively. Their comparison to reference strain profiles increased the accuracy of species assignment (Table 1 and Figure S1).

EPS producing isolates

Three isolates exhibited a negative EPS producing phenotype: S17, S29 and DSM2601. The isolates S5, S6, S9 and S10 resulted in high (+++) creamy colony phenotypes. The isolates S12 and S27 resulted in poor (+) liquid colony phenotypes. The isolates S13 and S14 did not succeed to grow under the test conditions.

Acidification kinetics

The 10 isolates were grown in BHI, a nutrient-rich broth, for 72h and pH was monitored every 5 min. Two profiles of acidification were observed by plotting dpH/dt as a function of time (Figure 1). *W. cibaria* and *Lactobacillus* spp. isolates exhibited a single acidification peak whereas *Leuconostoc* spp. isolates showed two peaks of acidification. From those profiles, several parameters were extracted: lag time, time for the first acidification peak (t_M), maximal acidification rate (V_M), pH at maximal rate (pH_M) and minimal pH observed during the culture (Table 2). These parameters were used as

variables for a principal component analysis, with isolates as observations (Figure 2). The two projection axes, F1 and F2, were representative of variables and accounted for 83.71%. Interestingly, the isolates grouped according to their genus. *Leuconostoc* spp. exhibited a long lag phase, a low V_M and a high minimal pH. Both *W. cibaria* and *Lactobacillus* spp. exhibited a short lag phase and a low minimal pH, but *W. cibaria* showed a high V_M contrarily to *Lactobacillus* spp.

One or two isolates of each genus were grown under the same conditions, but in MRS broth (Table 2). In this medium, the double peak profile was not observed for *Leuconostoc* spp. isolate. In MRS broth, maximal acidification rates were 2.4 to 4.5 fold higher than in BHI and minimal pH, pH_M and lag time were lower. Except for lag time, these parameters followed the same tendencies between isolates in BHI and in MRS broth.

Growth and adaptation to sub-lethal stress

An assay to evaluate isolate adaptation to different stress was standardized. The control condition showed that two isolates, S9 and S13, both *Leuconostoc citreum*, exhibited a poor growth in BHI over 48 h at 30°C, with an OD variation of less than 0.2 (Figure 3a). For other isolates, 48 h OD variation without stress was comprised between 0.4 and 0.9.

For these eight isolates, the stress adaptation assay was applied to evaluate adaptation to low pH, to high sodium chloride level or to oxidative stress, in respect to control condition (Figure 3b, c).

Oxidative stress adaptation was tested with two concentrations of hydrogen peroxide, 0.025% and 0.05% (Figure 3b). The lowest H_2O_2 level resulted for S12 and S27, both *W. cibaria* isolates, in a 40% decrease of 48h-growth compared to non-stressing condition, whereas other isolates growth did not significantly decrease. The effect of 0.05% H_2O_2 level was much more marked: growth of all isolates was affected but in different proportion. For instance, the effect of 0.05% H_2O_2 was more pronounced on the growth of S12 and DSM2601 (p-value<0.001) whereas the effect on S14 was not significant. A level of 0.07% H_2O_2 was tested, and none of the isolates was able to adapt to this concentration. Two isolates, S5 and S6, which belong to *Leuc. mesenteroides*, were particularly sensitive to pH, whereas S17 and S29, identified as *Lb. paraplantarum/pentosus* were the most

tolerant (Figure 3c). For *W. cibaria*, isolate S12, contrarily to isolate S27, was not able to grow in the presence of 5% NaCl (Figure 3c). The highest salt tolerance was observed for S5 (*Leuc. mesenteroides*), S10 and S14 (*Leuc. pseudomesenteroides*), DSM2601 and S27 (*W. cibaria*).

From the obtained data, four isolates demonstrated the best ability to adapt tested stress individually: S10 and S14 (*Leuc. pseudomesenteroides*), S17 (*Lactobacillus paraplantarum/pentosus*) and S27 (*W. cibaria*).

Pineapple antioxidant capacity modulation

Each isolate was inoculated into pasteurized pineapple juice and incubated for 48 h at 25°C with agitation. Pineapple juice exhibited an initial pH of 3.6 and an initial reducing sugar level of $94.4 \pm 10.0 \text{ g.L}^{-1}$. After 48h, no significant decrease in pH was observed and a significant growth was observed only for isolates S5, S12 and S17.

Antioxidant capacity was evaluated from seven different assays: ORAC, ABTS, hemolysis test, OD280nm measurement, DPPH, Folin-Ciocalteu, and LDL oxidation. Pasteurized juice was used as control.

From ORAC, ABTS, erythrocyte hemolysis, DPPH and OD 280nm assays, no significant difference could be observed between samples fermented with different isolates, neither between fermented juices versus control condition. The ORAC value for pineapple juice was $187 \pm 11 \text{ mM TE}$. For ABTS assay, pineapple juice value was $57.0 \pm 3.2 \text{ mM TE}$. Hemolysis test showed a HT50 (time for half-hemolysis) value of $103 \pm 49 \text{ min}$ for 100-fold diluted juice, and DPPH assay resulted in a value of $42.6 \pm 2.1 \text{ g GAE.L}^{-1}$ for control juice. The measurement of OD at 280 nm of pineapple juice resulted in $300 \pm 18 \text{ g GAE.L}^{-1}$.

Contrary to the above, Folin-Ciocalteu and LDL oxidation assays showed a number of variations (Figure 4).

Folin-Ciocalteu assay resulted in $1.15 \pm 0.05 \text{ g GAE.L}^{-1}$ for pineapple juice. Pineapple juice fermented with isolate S14 (*Leuc. pseudomesenteroides*) exhibited a Folin-Ciocalteu value of $1.43 \pm 0.25 \text{ g}$

GAE.L⁻¹. The two values significantly differed with a p-value of 0.06. Other fermented juices did not differ from the control.

From LDL oxidation assay, the determination of V50, the time for half-LDL oxidation, was used to compare samples and control. Distilled water was used as control and showed a V50 value of 56.6 ± 0.8 min. A 10⁴-fold diluted juice did not exhibit a significant difference compared to water, but the use of 10³-fold diluted juice resulted in an absence of measurable oxidation of LDL by copper. Only the juice fermented with S27 (*W. cibaria*) and 10⁴-fold diluted exhibited a significant delayed oxidation versus control with a V50 value of 59.7 ± 1.6 min. The other diluted fermented juice did not differ from the control.

DISCUSSION

The interest in fruit or vegetable foods, and particularly in lactic fermented foods, has considerably increased over the last decade as these foods can significantly contribute to a healthy diet. In the present study, lactic acid bacteria were isolated from papaya, tomato or sliced cabbage samples, identified by molecular method and characterized for phenotypic traits related to technological properties relevant for food fermentation. A special focus was made on antioxidant activity, choosing an acidic fruit juice and a short incubation time for testing.

The sampled fruits and vegetables exhibited very diverse levels of LAB, with population ranging between 5.1 and 10.0 cfu.g⁻¹. The highest population was observed for sliced cabbage, a ready-to-eat vegetable, for which cutting resulted in increased surface and nutrient availability. We limited our study to 10 isolates chosen from morphological diversity criterion, and those isolates were showed to belong to five different species and three genera. Whereas *Weissella* spp. and *Leuconostoc* spp. are frequently isolated from fermented foods, starters used in vegetable food fermentation essentially belong to *Lactobacillus* spp. Interestingly, isolates exhibited very different fermentation kinetics parameters. The use of acidification kinetics as a tool to monitor fermentation performance has been developed more than 25 years ago (Spinnler and Corrieu 1989) and has been widely applied to

characterize dairy starters (Cachon et al. 2002; Latrille et al. 1992; Pinheiro De Souza Oliveira et al. 2009; Xanthopoulos et al. 2001). However, this tool has never been previously used to characterize *Leuconostoc* spp. or *Weissella* spp. Our study revealed that *Weissella cibaria* can act as a potent acidifier. No significant decrease of pH was observed during short-term fermentation of pineapple juice, probably as a consequence of the low initial pH (3.6). Dairy bacteria grown in milk exhibited V_M values that range between 0.2 and 1.2 UpH.h⁻¹ and T_M between 3 and 10h (Cachon et al. 2002; Latrille et al. 1992; Pinheiro De Souza Oliveira et al. 2009; Xanthopoulos et al. 2001). In our study, the values of V_M on MRS broth ranged between 0.10 and 0.15 UpH.h⁻¹ whatever the species, which is by far much lower than V_M observed in dairy studies. MRS broth is recommended for LAB culture: as expected, even lower V_M were observed in BHI. Interestingly, acidification kinetics in BHI showed genus related patterns, hereby characterized by V_M , t_M , pH_M , minimal pH and lag time. Differences between slow and rapid fermentative isolates and between high and low acidifiers were easily pointed out in this medium.

The comparison of growth yield between un-stressing and stressing conditions is critical since fermentation generally results from bacterial competition in a stressing environment (Serrazanetti et al. 2013). Indeed, most fruits and many vegetables exhibit a low pH. Moreover, addition of salt in vegetable preparations is often used as a selective agent. Stress adaptation ability widely differed between isolates, even within the same species. Interestingly, S12 and S27, both *W. cibaria*, shared similar (GTG)₅ profile, but showed different stress adaptation ability. On the contrary, S17 and S29, both *Lb. paraplantarum/pentosus*, shared similar (GTG)₅ profile and showed same adaptation patterns. Several mechanisms are involved in LAB stress tolerance: glutathione system (Kim et al. 2012; Zhang and Li 2013), the so-called general stress response or acid tolerance response (ATR) (van de Guchte et al. 2002), and the ability to detoxify reactive oxygen species, thanks to antioxidant enzymes (An et al. 2011; Bruno-Bárcena et al. 2010; Ramesh et al. 2011). Although LAB are catalase negative, other enzymes, such as superoxide dismutase, peroxidases or NADH oxidases might be

involved in detoxification. These key-activities deserve to be determined in the most resistant isolates.

Recently, a relationship between redox regulation mechanisms and production of exopolysaccharides (EPS) was shown in *Lactobacillus casei* (Zhang and Li 2013). Moreover, many EPS, produced by *Leuconostoc* spp., were shown to exhibit antioxidant activities by themselves (Li et al. 2014; Pan and Mei 2010). As *Weissella* spp. is a high EPS producer, investigation of the activities of enzymes involved in intracellular redox balance and oxidative stress detoxification is of particular importance for this genus.

The contribution of a short fermentation time to the antioxidant capacity of pineapple juice was poor. Only two antioxidant capacity tests, Folin-Ciocalteu and LDL oxidation assays, showed significant differences between fermented juice and control. Noteworthy enough, this fermentation step did not result in a loss of antioxidant capacity of juice. Other studies have pointed differences in antioxidant capacity after fermentation. For instance, in olives, in green or red smoothies or in pomegranate juice, fermentation with *Lb. plantarum*, alone or associated with *W. cibaria* or *Pediococcus pentosaceus*, decreased the antioxidant capacity (Di Cagno et al. 2011; Filannino et al. 2013; Othman et al. 2009). In other studies, over a very short time (8-17h) of fermentation of tempeh (soybean seeds) or tomato juice with *Lb. plantarum*, the antioxidant capacity was maintained (Di Cagno et al. 2009; Starzyńska-Janiszewska et al. 2014). Conversely, fermentation of white cabbage over 7 days, with *Lb. plantarum* and/or *Leuc. mesenteroides*, showed a two-fold increase in the antioxidant capacity measured with ORAC (Martinez-Villaluenga et al. 2012). Similarly, the antioxidant activity of soymilk increased by three-fold after 24h fermentation with a *Lb. rhamnosus* starter (Marazza et al. 2012). The relationship between antioxidant capacity modulation and changes in composition or enzyme activities deserve to be extensively investigated.

Based on stress tolerance, four isolates appeared resistant: S10, S14, S17 and S27. They were distributed over the four species identified. All four isolates revealed very different acidification kinetics. Among those, only S14 and S27 modulated significantly the juice antioxidant capacity. The

applied method is thus relevant to identify potential starters which may be used to perform mild or strong, slow or rapid food fermentation. A larger screening of isolates, completed with enzymatic activity screening, could highlight relationships between stress resistance, ability to modulate antioxidant capacity and enzyme activities.

Isolate S14 belong to *Leuc. pseudomesenteroides* species and isolate S27 to *W. cibaria*. These species are commonly identified from fermented vegetables, including cabbage and olive (Di Cagno et al. 2013). However, their use as starters is not yet described. In addition, the physiological properties of *Weissella* spp. are still poorly known, but recently niche specific features were highlighted at genomic level (Lynch et al. 2015). The S27 isolate showed in this study a high potential for its use as starter for fermented fruit or vegetable juices. An experimental plan with different fruit juices and different fermentation durations is warranted in order to determine the safety and the sensorial properties of the resulting food.

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TABLES

Table 1: Origin and identification of isolates

| Isolate | Origin | Numeration (cfu.g ⁻¹) | 16S partial sequence (bp) | % ID | Species | (GTG)5 profile |
|----------|-----------------|-----------------------------------|---------------------------|----------------------------|--|-----------------------------------|
| S5 | Papaya | 1.33 10 ⁵ | 430 | 99% LN774434.1 | <i>Leuc. mesenteroides/pseudomesenteroides</i> | <i>Leuc. mesenteroides</i> |
| S6 | Papaya | 1.33 10 ⁵ | 939 | 99% KJ477421.1 | <i>Leuc. mesenteroides/pseudomesenteroides</i> | <i>Leuc. mesenteroides</i> |
| S9 | Papaya | 1.33 10 ⁵ | 941 | 99% AB904775.1; HF562952.1 | <i>Leuc. holzapfelii/citreum</i> | <i>Leuc. citreum</i> |
| S10 | Papaya | 1.33 10 ⁵ | 945 | 99% GQ351323.1 | <i>Leuc. mesenteroides/pseudomesenteroides</i> | ND* |
| S13 | Tomato | 6.64 10 ⁵ | 915 | 99% KF879147.1; AB904775.1 | <i>Leuc. holzapfelii/citreum</i> | <i>Leuc. citreum</i> |
| S14 | Sliced cabbage | 1.57 10 ⁹ | 921 | 99% KF879168.1 | <i>Leuc. mesenteroides/pseudomesenteroides</i> | ND |
| S12 | Papaya | 1.33 10 ⁵ | 935 | 99% AB761300.1 | <i>W. cibaria/confusa</i> | <i>W. cibaria</i> |
| S27 | Sliced cabbage | 1.07 10 ¹⁰ | 879 | 100% HF562959 | <i>W. cibaria/confusa</i> | <i>W. cibaria</i> |
| S17 | Papaya | 1.30 10 ⁵ | 879 | 99% KJ690749.1; KJ690750.1 | <i>Lb. plantarum/paraplantarum/pentosus</i> | <i>Lb. paraplantarum/pentosus</i> |
| S29 | Tomato | 6.64 10 ⁵ | 614 | 99% KC914585.1; KP189214.1 | <i>Lb. plantarum/paraplantarum/pentosus</i> | <i>Lb. paraplantarum/pentosus</i> |
| DSM 2601 | Pickled cabbage | - | | | <i>Lb. plantarum</i> | |

* ND: not determined

Table 2: Parameters of acidification (mean \pm standard deviation)

| Isolate | Lag time (h) | | pH min | | V_M (mU pH.h ⁻¹) | | pH _M | | t _M (h) | |
|---|--------------|------------|--------|-----------|--------------------------------|---------|-----------------|-----------|--------------------|------------|
| Growth in BHI (initial pH 6.8) | | | | | | | | | | |
| S5 | 11.42 | \pm 3.42 | 5.9 | \pm 0.1 | 32 | \pm 1 | 6.3 | \pm 0.2 | 4.29 | \pm 0.41 |
| S6 | 10.92 | \pm 0.59 | 5.8 | \pm 0.1 | 33 | \pm 1 | 6.3 | \pm 0.1 | 4.25 | \pm 0.24 |
| S9 | 5.13 | \pm 0.77 | 5.9 | \pm 0.1 | 38 | \pm 5 | 6.4 | \pm 0.1 | 4.00 | \pm 0.59 |
| S10 | 4.17 | \pm 0.59 | 6.2 | \pm 0.2 | 38 | \pm 7 | 6.4 | \pm 0.1 | 3.00 | \pm 0.47 |
| S13 | 19.58 | \pm 0.59 | 6.2 | \pm 0.4 | 26 | \pm 9 | 6.5 | \pm 0.3 | 4.75 | \pm 1.71 |
| S14 | 17.75 | \pm 0.59 | 6.1 | \pm 0.1 | 31 | \pm 6 | 6.4 | \pm 0.1 | 5.58 | \pm 0.59 |
| S12 | 6.58 | \pm 2.24 | 4.9 | \pm 0.1 | 66 | \pm 3 | 6.1 | \pm 0.1 | 5.13 | \pm 0.12 |
| S27 | 2.04 | \pm 1.24 | 4.9 | \pm 0.1 | 50 | \pm 1 | 6.1 | \pm 0.6 | 6.25 | \pm 1.36 |
| S17 | 10.96 | \pm 0.06 | 5.6 | \pm 0.1 | 43 | \pm 1 | 6.2 | \pm 0.1 | 11.29 | \pm 0.35 |
| S29 | 11.54 | \pm 1.00 | 5.7 | \pm 0.1 | 43 | \pm 7 | 5.7 | \pm 0.1 | 10.79 | \pm 0.41 |
| DSM2601 | 8.13 | \pm 0.65 | 4.5 | \pm 0.1 | 33 | \pm 1 | 5.8 | \pm 0.1 | 12.38 | \pm 1.47 |
| Growth in MRS broth (initial pH 5.7) | | | | | | | | | | |
| S5 | 4.92 | | 4.1 | | 118 | | 5.1 | | 5.33 | |
| S10 | 6.67 | | 4.2 | | 105 | | 5.0 | | 8.33 | |
| S27 | 3.17 | | 3.7 | | 156 | | 4.8 | | 10.08 | |
| DSM2601 | 3.75 | | 3.7 | | 150 | | 4.8 | | 10.50 | |

FIGURE LEGENDS

Figure 1

Acidification profile (dpH/dt) obtained from three isolates grown in BHI (initial pH 6.8) at 37°C. Black line: S12 *W. cibaria*; Grey line: S17 *Lb. plantarum/pentosus*; Bold line: S13 *Leuc. holzapfelii/citreum*

Figure 2

Principal component analysis of isolates (observations). Variables are acidification profile parameters: Tm, Vm, pHm, pHmin and lag time.

Figure 3

Growth of isolates over 48h at 30°C. A: OD 600nm variation in BHI; B: relative OD 600nm variation in the presence of oxidative stress. Grey bar: H₂O₂ 0.025%; black bar: H₂O₂ 0.05%. C: relative OD 600nm variation in the presence of acid (pH 4.5) or salt (5% NaCl) stress. Grey bar: pH 4.5; black bar: NaCl 5%. *p<0.05; **p<0.01; ***p<0,001 compared to control condition (BHI, 100%).

Figure 4

Antioxydant activity of pineapple juice after 48h incubation at 25°C, without or with indicated isolate. A: Folin-Ciocalteau values, expressed as GAE g.L⁻¹; B: LDL oxidation kinetics for isolate S27. V50 values are shown by arrows. **p<0.01 compared to control condition (juice).

Figure S1

Dendrogram for *Weissella* spp isolates and reference strains using (GTG)₅ genotyping.

See materials and methods for cluster analysis and tree building.

Figure 1

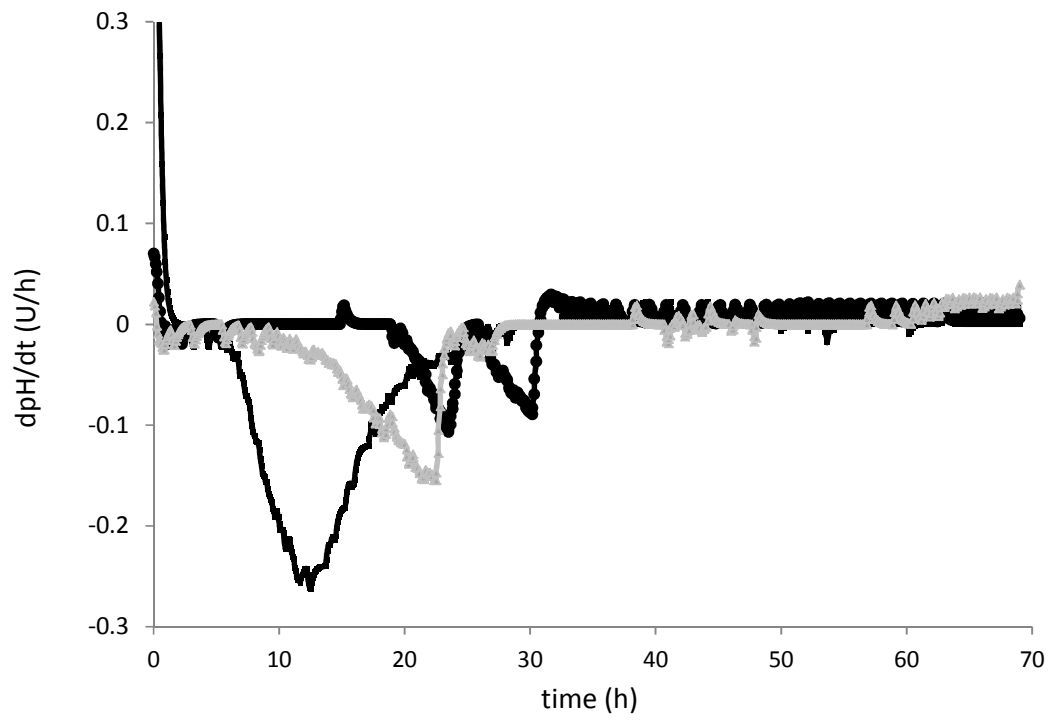


Figure 2

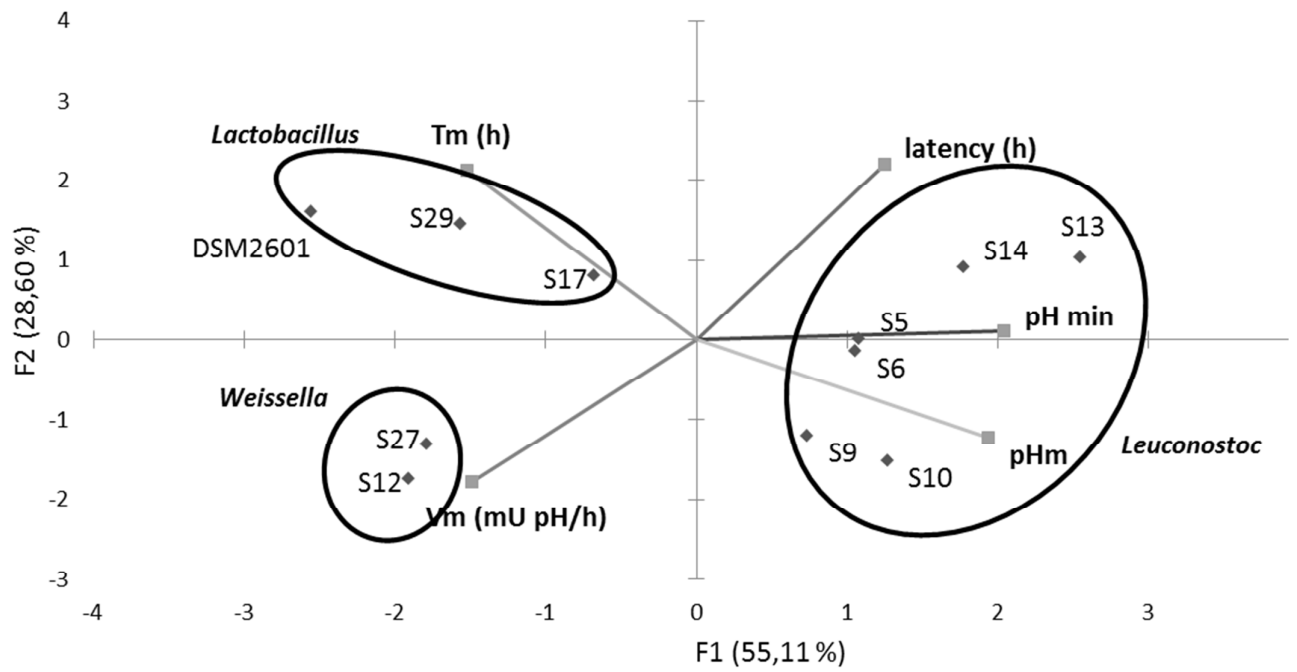
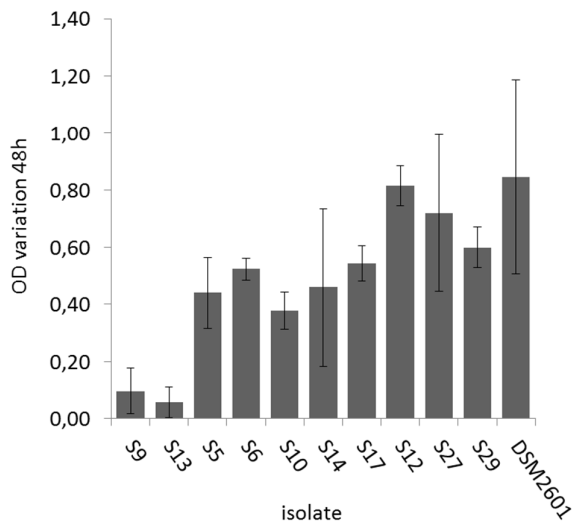
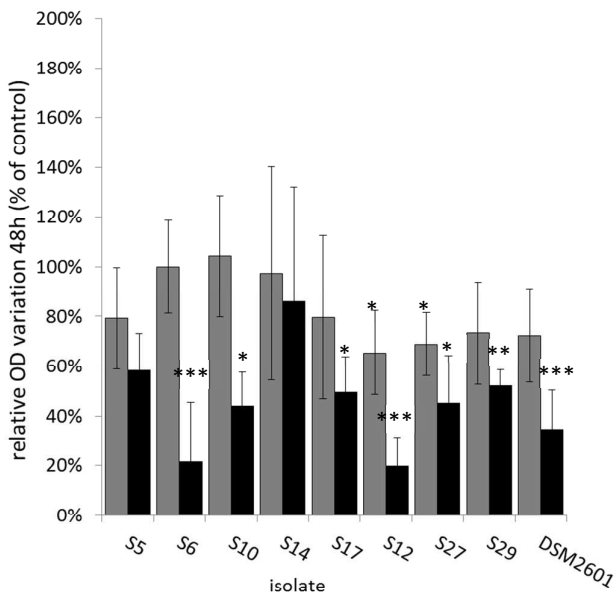


Figure 3

A



B



C

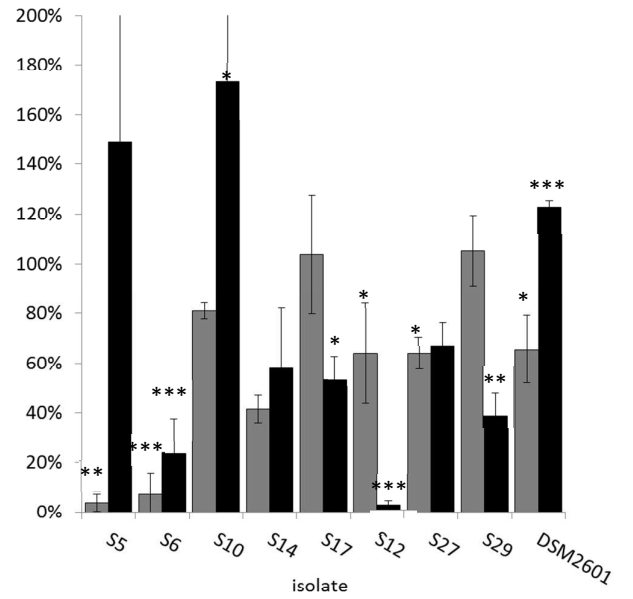
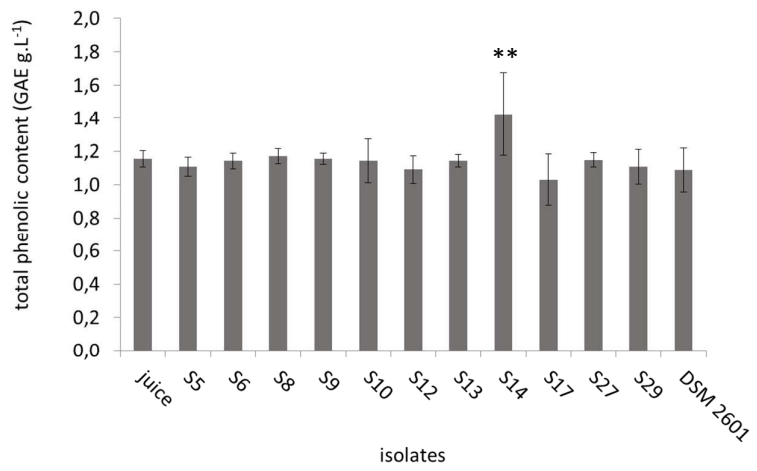


Figure 4

A



B

