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Selection of *Lactobacillus paracasei* strains from grated carrots by-products for L-lactic acid production

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ABSTRACT

Beside its utilisation as food preservative, lactic acid can also be used to make biopolymers, or to enter in the composition of cleaning products. Nowadays, different ways are explored to optimize its production. The use of vegetable wastes could appear as a profitable option to reduce costs. In this work, 40 different native lactic acid bacteria isolated from fermented carrot pulps were analysed and compared for their ability to produce lactic acid. The selection relied on the use of two model media, the synthetic Man Rogosa and Sharpe (MRS) medium and a sterilized carrot-like medium. This allowed for the isolation of two interesting species: *Lactobacillus paracasei* and *Lactobacillus harbinensis*, which produced high concentrations of L-lactic acid in MRS broth. However, the yield obtained with the carrot-like medium was less important and will have to be optimized in the future. *L. paracasei* proved to be more interesting than *L. harbinensis*. In particular, the strain A1L was able to metabolize starch. This ability could be a selective advantage in a complex vegetable matrix to overwhelm the other adventitious microorganisms.

INTRODUCTION

In 2013, 1.6 billion tonnes of wastes were generated, 15 to 25% being from vegetable origins (FAO, 2013). At present, different methods are used to valorise these by-products; for instance, composting, animal feed or anaerobic digestion to produce methane. At the same time, many studies focus on the production of interesting biomolecules by fermentation processes, which could be used, for example in cosmetics or in medicine (López-Gómez et al., 2019). These compounds include organic acids, enzymes, or aroma molecules (Abu Yazid et al., 2017). However, the composition of vegetable matrix varies a lot depending on their degradation state, adventitious microbes inside or harvest conditions. Furthermore, during the optimization step, the type of vegetable and its biochemical composition are key factors to produce compounds and to obtain good yields. Now, it is essential to find new options to replace synthetic culture media and to avoid using agriculture plots to produce chemical molecules.

Among the different native microbes which can be found in and on raw vegetables, lactic acid bacteria (LAB) population frequently dominates. These bacteria have been used for centuries to ferment different substrates, spontaneously or in a controlled way. For instance, LAB are used to make fermented food products; among them, olives, kimchi or pickles are often referred to in the literature. The LAB microflora of these food products includes different genera, *Lactobacillus*, *Leuconostoc*, *Weissella*and *Pediococcus*. If homofermentative LAB produce only lactic acid, heterofermentative LAB also release acetic acid, CO_2 and ethanol, depending on the

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type of carbohydrate metabolized and the physico-chemical environment (Gänzle, 2015). In recent years, L-lactic acid has become more and more interesting. It is used to synthesize poly(lactic acid) substances available for bioplastic production (Abdel-Rahman et al., 2013).

Our project was undertaken to investigate the fermentation aptitude of carrot pulps to produce L-lactic acid. The carrot pulps resulted from the peeling step during the industrial production of grated carrots Two strategies can be followed: 1- the utilisation of type LAB strains or 2- the direct isolation of microorganisms from the vegetable wastes which will be further used as substrate (Di Cagno et al., 2013; Gardner et al., 2001). We decided to focus on this second way, considering that wild bacteria are supposed to be more adapted to their native environment. Di Cagno et al. (2008), for instance fermented three different vegetable matrix (carrot, marrows and French beans). They observed that autochthonous LAB was able to grow at higher levels than allochthones LAB, to reach around 9.2 log CFU/mL. The acidification ability of these autochthonous microbes was also more intense. As a consequence, higher lactic acid rates were obtained. As highlighted in this study, and because of the tremendous amounts of by-products generated by the food industry, carrots pulps could be used as substrate to support LAB growth. To release lactic acid, LAB need an available carbon source. Alasalvar et al. (2001) detected sucrose, glucose and fructose in carrots by HPLC –around 2.69, 1.44 and 1.34 g/100 g, respectively. This substrate appears so convenient for microbial cultures.

Based on a previous study made on carrot pulps (Godard et al., 2018), in the present work, we decided to select autochthonous LAB. We intend to use them as starters to valuate this by-product to obtain high quantities of lactic acid.

MATERIALS AND METHODS

LAB isolation

LAB were chosen and isolated from Man Rogosa and Sharpe (MRS) agar on the basis of their morphotypes (Biokar diagnostics, France) after culture of fermented carrot pulp samples. These products originated from a food plant located in the Region of Lyon (France). They have been incubated at 24 and 37°C in Erlenmeyer flasks (320 g/flask) for 9 days, as described by Godard et al. (2018). MRS petri dishes were incubated at 30°C in anaerobic condition (GENbag Anaerobic, Biomérieux, Marcy l'Etoile, France), for 48 h. Bacterial colonies were picked, purified on MRS agar and tested for the presence of the catalase. Only the catalase negative isolates were reserved. A sample of an overnight culture (MRS broth, 30°C) was then put in glycerol (15%, v/v, Roth) to be kept at -80°C.

16S rRNA gene sequencing

Total DNA of the 40 isolate used in this work was extracted from a dense suspension of bacteria obtained from several colonies mixed together in 1 mL of pure water (Godard et al., 2018). The 16S rRNA gene (~1500 bp) was amplified using the universal primers W02R (5'-GNTACCTTGTTACGACTT-3') and W18F (5'-GAGTTTGATCMTGGCTCAG-3') (Sigma, Germany). The Polymerase Chain Reaction (PCR) reaction medium included 12.5 µL of Phusion High-Fidelity PCR Master Mix (Thermo Scientific), 1.25 µL of each primer (0.5 µM), 5 µL of pure DNA and 5 µL of distilled water. Amplifications were performed using the PCR cycle described by Schlusselhuber et al. (2018) (T100 Thermal Cycler, Bio-Rad, USA). DNA sequences were analysed by BIOFIDAL (France). Sequences were then compared to GenBank database (NCBI). Alignments of 16S rRNA gene sequences were achieved with the Muscle Alignment tool of the MEGA 7 software. Evolutionary distances were computed according to the Tamura-Nei method. For the LAB group assignment, we followed the classification proposed by Salvetti et al. (2012) and Gu et al.(2013).

Screening of isolated LAB for lactic acid production

Isolates were screened in microplates (96 wells) with modified-MRS medium (mMRS): glucose (20 g/L, Sigma), polypeptone (10 g/L, Oxoid), meat extract (10 g/L, Biokar), autolytic yeast extract (5 g/L, Biokar), tween 80 (1.08 g/L, AppliChem PanReac), sodium acetate (5 g/L, Sigma), ammonium citrate (2 g/L, Honeywell, USA), magnesium sulfate (0.2 g/L, Sigma), manganese sulfate (0.05 g/L, Sigma), bromocresol green (0.004 g/L, Chimie-Plus Laboratoires), adjusted to pH 6.4. After thawing, 10 mL of MRS broth were inoculated with one strain and incubated at 30°C. The optical density at 600 nm (OD_{600}) of the overnight culture was then adjusted to 1. In each well of the microplate, 180 µL of sterile mMRS medium were added with 20 µL of the adjusted suspension. Acidification efficiency was evaluated after a 4.5 h delay. This time, sufficient to discriminate the samples from each other, was based on the shift of the pH indicator. The most effective strains were kept for further tests.

Then, strains previously selected were tested in Erlenmeyer flasks. LAB cultures were first carried out in 10 mL of MRS broth (30°C, one night). The OD_{600} of each culture was adjusted to 0.1 in MRS broth to be cultured at 30°C. As soon as the OD_{600} reached 0.3 (exponential phase, corresponding to 8 log CFU/mL), the Erlenmeyer flasks containing either MRS broth (150 mL) or carrot like

medium (150 g) were inoculated at OD_{600} of 0.01 (for description of the carrot like medium, see below). Fermentations were carried out for 72 h at 30°C.

For the MRS medium, supernatants were recovered after a centrifugation step of 5 min at 20,000 g. For carrot-like medium, 2 g of carrot pulps were suspended in 18 mL of sterile physiological water (NaCl, 9 g/L, Sigma) and homogenised with a stomacher apparatus (Smasher®, Biomérieux, France).

Carrot-like medium characterization

Fermentations carried out in the carrot-like medium were performed in triplicate at 30°C for 72 h. Supernatants samples were stored at -20°C prior any analysis. Concentrations of acetic and lactic acids were measured with enzymatic kits (R-Biophram, Germany). The total titratable acidity (TTA) value was defined as the amount of a 0.1 N NaOH solution required to reach a pH-value of 8.5. The results were expressed in microliters (Rizzello et al., 2018).

Carrot pulps were retrieved from the same producer stated above and stored at -20°C. The composition of the carrot-like medium was the following (for 100 g): 75 g of mashed carrots formerly cooked at 100°C for 30 min, 25 g of water, 0.4 mg of bromocresol green; the pH was adjusted at pH 6.4. The medium was then heat-treated at 100°C for 30 min.

pH was determined with a pH-meter (HI 2211, pH/ORP Meter, Hanna instruments), on 10 mL of the supernatant. Water availability (A_w) was measured with a A_w meter (Aw Sprint, TH-500, Novasina) on a sample of the carrot-like medium.

$(GTG)_5$ LAB profile

The comparison of LAB isolates was performed by $(GTG)_5$-PCR. PCR mix contained: 2 µL 10X Reaction Buffer, 4 µL $(GTG)_5$ primer (5′-GTG GTG GTGGTG GTG-3′), 1 µL dNTP (20 mM), 0.6 µL $MgCl_2$ (50 mM), Taq DNA polymerase (5 units/µL, Sigma), 15.2 µL of water and 1 µL of diluted DNA (1/10). All reagents came from Eurogentec (Belgium). The initial denaturation at 94°C for 5 min was followed by 32 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and extension at 72°C for 1 min.

The last cycle was followed by the final elongation at 72°C for 10 min. The band patterns were normalized and processed using GelCompar 3.1 software (Applied Maths, Kortrijk, Belgium) as previously described by Demarigny (2012). To ensure reproducible $(GTG)_5$-PCR results, total DNA from two strains was prepared independently six times and essayed by $(GTG)_5$-PCR. All profiles of a strain had similarity coefficient higher or equivalent to 80%.

Carbohydrate utilization profile

The four selected strains for lactic acid production were tested according to biochemical tests based on the fermentation of 49 different sugars using an API 50 CHL *Lactobacillus* system (Biomérieux, Marcy l'Etoile, France). Kits were inoculated and incubated according to the manufacturer's procedures.

Statistical analyses

Statistical analyses were performed using the XLStat software (Microsoft, 2016).

RESULTS

LAB diversity in fermented carrot pulps

According to the results we obtained in a previous study (Godard et al., 2018), LAB population dominated over the other microflora enumerated in the carrot pulps. The LAB levels ranged between 8.20 and 9.07 log CFU/mL following the samples used for the isolation steps. Yeasts and fungi, enterococci and acetic acid bacteria were counted at sub-dominant levels, ranging between 5.35 and 7.22 log CFU/mL. *Enterobacteriaceae*, *Bacillus* and *Pseudomonas* never exceed 5.06 log CFU/mL. Forty colonies were isolated from MRS agar cultured in anaerobic conditions. All the isolates proved to be catalase negative, a feature characteristic of LAB. Among them, two genera were identified according to the 16S rRNA gene sequencing: *Lactobacillus* and *Leuconostoc*. Seventeen species were identified: *L. paracasei* (7 out of 40 isolates), *Lactobacillus nenjiangensis* (6/40), *L. harbinensis* (4/40), *Lactobacillus coryniformis* (3/40), *Lactobacillus parabuchneri* (3/40), *Leuconostoc kimchii* (3/40), *Lactobacillus sunkii* (2/40), *Lactobacillus mudanjiangensis* (2/40), *Lactobacillus delbrueckii* (2/40), *Lactobacillus silogincola* (1/40), *Lactobacillus rhamnosus* (1/40), *Lactobacillus porcinae* (1/40), *Lactobacillus plantarum* (1/40), *Lactobacillus perolens* (1/40), *Lactobacillus otakiensis* (1/40), *Lactobacillus koreensis* (1/40) and *Lactobacillus bifermentans* (1/40) (Figure 1). The phylogenetic relationships between LAB strains, based on their 16S rRNA gene sequencing, were represented on Figure 2. Nine groups of *Lactobacillus* were identified.

Screening of LAB for lactic acid production

Among the 40 strains, 31 were able to grow in MRS broth

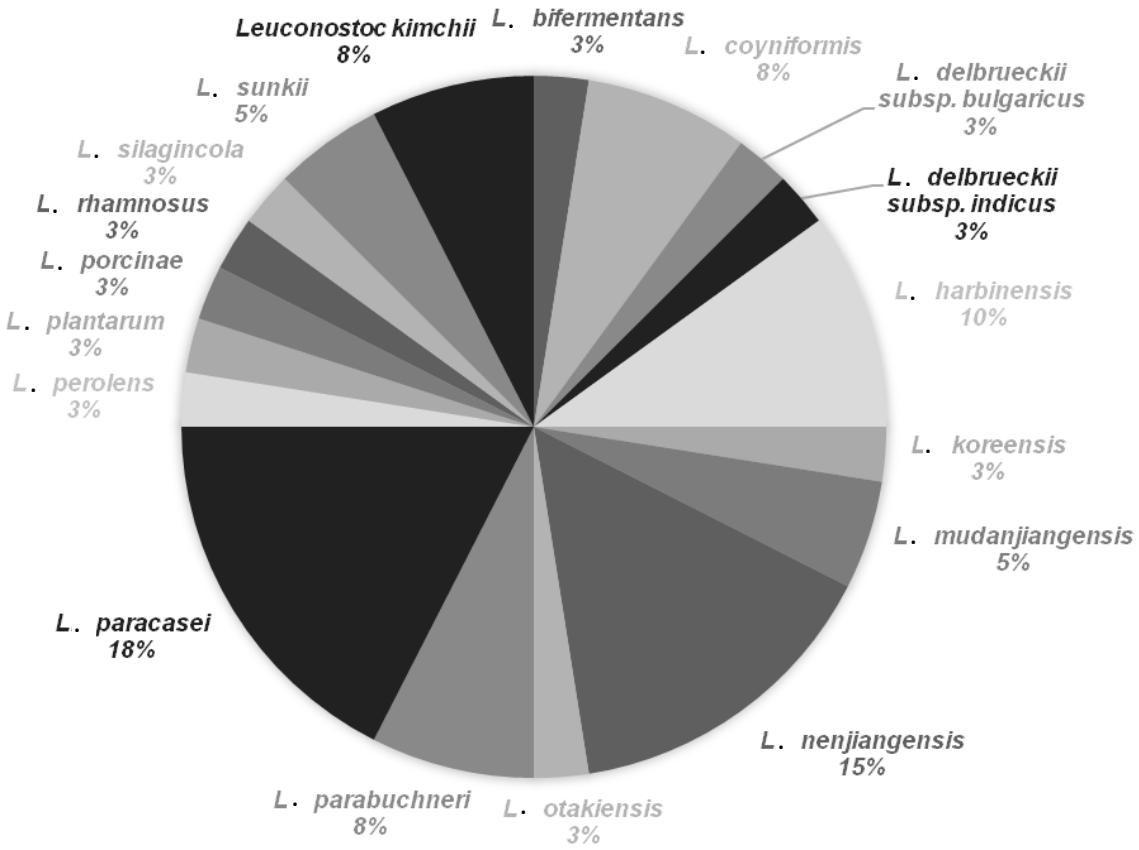

Figure 1. Diversity of the 40 strains isolated from fermented carrot pulps based on the 16S rRNA gene sequencing. Stains were picked on MRS agar incubated in anaerobic conditions for 72 h at 30°C.

after an overnight incubation at 30°C. Their acidification ability was tested in microplates in mMRS medium. After a 4.5 h delay, nine strains changed quickly the pH indicator from blue to yellow. The other strains were put aside for the rest of the study. The nine strains belonged to the species *L. paracasei* (2), *L. nenjiangensis* (1), *L. harbinensis* (2), *L. coryniformis* (1), *Lactobacillus mudajiangensis* (1), *L. porcinae* (1), *L. kimchii* (1). D- and L-lactic acid concentrations produced by the nine LAB strains were measured in Erlenmeyer flasks containing either 150 mL of MRS broth or 150 g of carrot-like medium (approximately 150 mL). The mean concentrations measured were respectively equal to 998 mg/L and 628 mg/L in carrot-like medium and 13,721.2 mg/L and 14,952.7 mg/L in MRS medium. In detail, lactic acid rates measured in the carrot-like medium after 72 h varied from 225 to 1,008 mg/L. The L-lactic acid proportion ranged between 15 and 98% depending on the strains. On the basis of the D and L-lactic acid rates obtained (materialized by the outlined variables on Figure 3), a principal component analysis was performed. The first and the second axes of the PCA totalized 90.11% of the information inertia. B1L, F3L and F5L strains produced lesser amounts of D- and L-lactic acid on carrot-like medium (on the left of the Figure 3). They were not kept afterwards. The two species of *L. paracasei* (A1L and F7L) and *L. harbinensis* (E6L and A9L), placed on the right of the figure, were able to produce high lactic acid concentrations. For these four strains, L-lactic acid accounted for more than 94% of the total lactic acid produced.

Carrot-like medium characterization

Forty-nine carbohydrates were tested to determine the fermentative profiles of the strains; nine allowed us to discriminate the two *L. paracasei* (A1L and F7L) and the two *L. harbinensis* (E6L and A9L) (Table 1). In particular, strain A1L used dulcitol and starch, and strain F7L, D-sorbitol and D-adonitol. Concerning the two *L. harbinensis* strains, some differences were also observed. For instance, A9L strain was able to metabolize D-lactose, unlike E6L strain which specifically used D-arabinose and D-sorbitol. $(GTG)_5$-PCR profiles allowed us to separate the strains. At the similarity

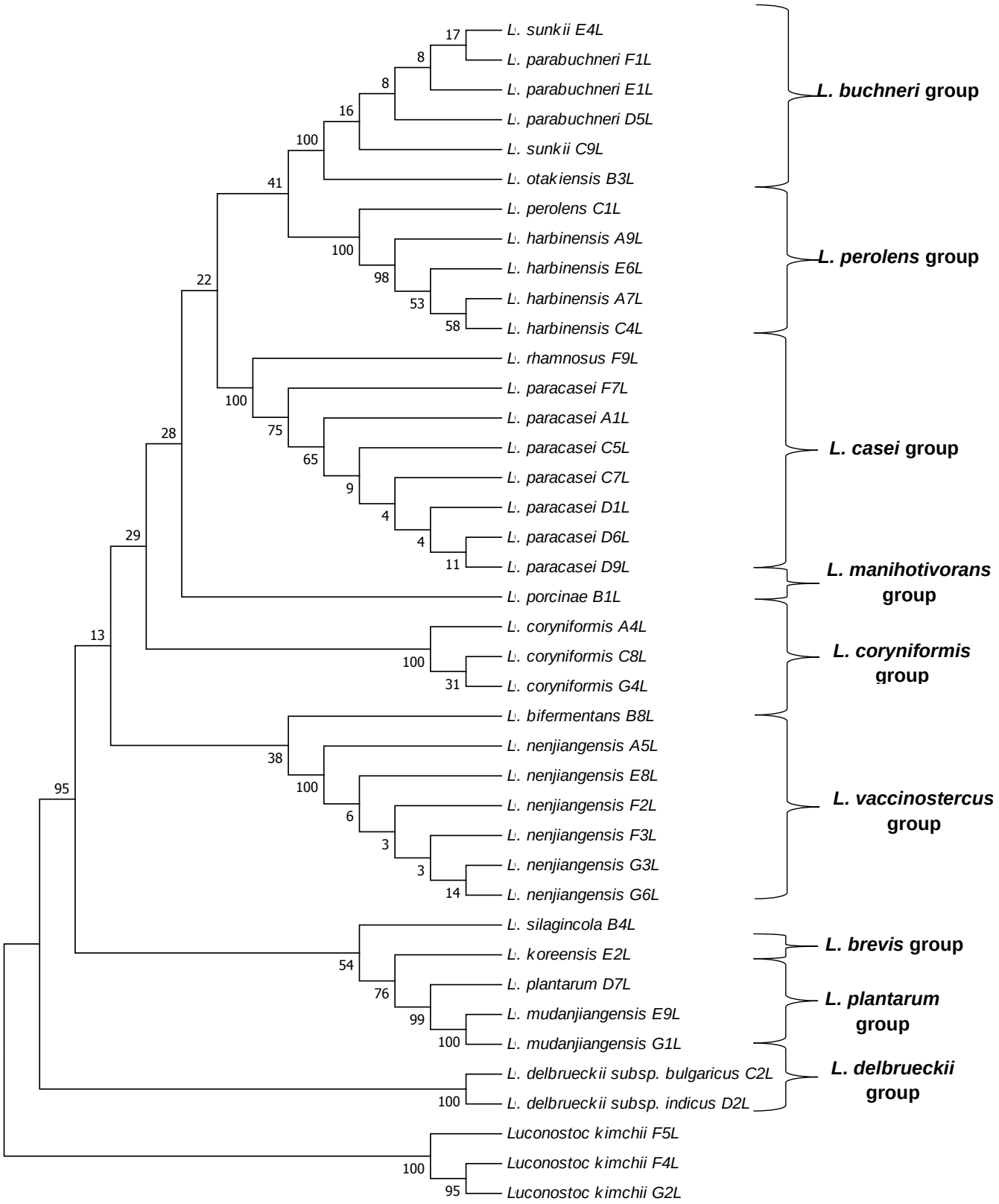

Figure 2. Phylogenetic tree of the LAB 16S rRNA genes isolated from carrot pulps based on the Neighbour-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Tamura-Nei method.

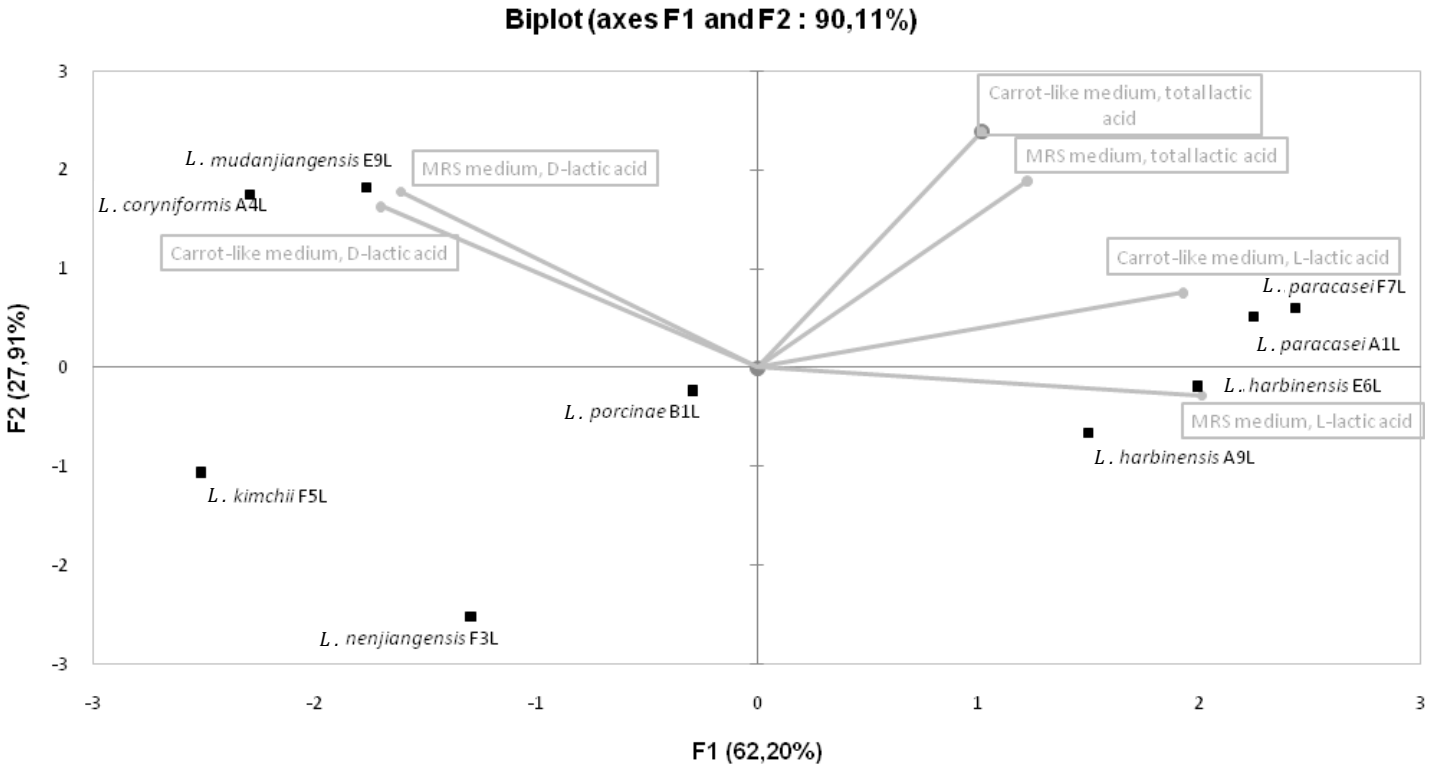

Figure 3. Principal Component Analysis (PCA) of D-, L- and total lactic acid rates. Results were obtained after MRS and carrot-like media fermentation for 72 h at 30°C. Strains able to acidifying mMRS medium after a 4.5 h delay, are figured in plain squares; and variables in plain circles and surrounded.

Table 1. Carbohydrate profiles of *L. paracasei* A1L and F7L and *L. harbinensis* A9L and E6L. Absence of growth: -, growth: +, weak growth: ±.

Substrate	*L. paracasei* A1L	*L. paracasei* F7L	*L. harbinensis* A9L	*L. harbinensis* E6L
D-Arabinose	-	-	-	±
L-Arabinose	-	-	+	+
D-Adonitol	-	+	-	-
Dulcitol	+	-	-	-
D-Mannitol	+	+	-	-
D-Sorbitol	±	+	-	±
D-Lactose	+	+	+	-
D-Melibiose	-	-	+	±
Starch	±	-	±	±

coefficient of 80%, the four strains finally selected appeared clearly distinct (Figure 4).

Environmental parameters, especially pH and A_W, are crucial for microbial growth. In the carrot-like medium, the pH, the TTA and the Aw at the beginning of the fermentation step were respectively equal to 5.30 ± 0.07 and 127.50 ± 10.55 µL and 0.994 ± 0.001. After 72 h of incubation, the carrot-like medium seeded with *L. paracasei* A1L or F7L strains was more acid than the media containing *L. harbinensis* A9L or E6L strains (Table 2). The initial pH measured for all samples was equal to 5.30 ± 0.07. pH variations measured were equal to -1.67 ± 0.23 and -1.80 ± 0.08, and -1.22 ± 0.03 and -1,40 ± 0.03, respectively. Thereafter, as a consequence of the fermentation step, the pH decreased to reach respectively 3.57 ± 0.13 and 3.53 ± 0.07, and 4.10 ± 0.02

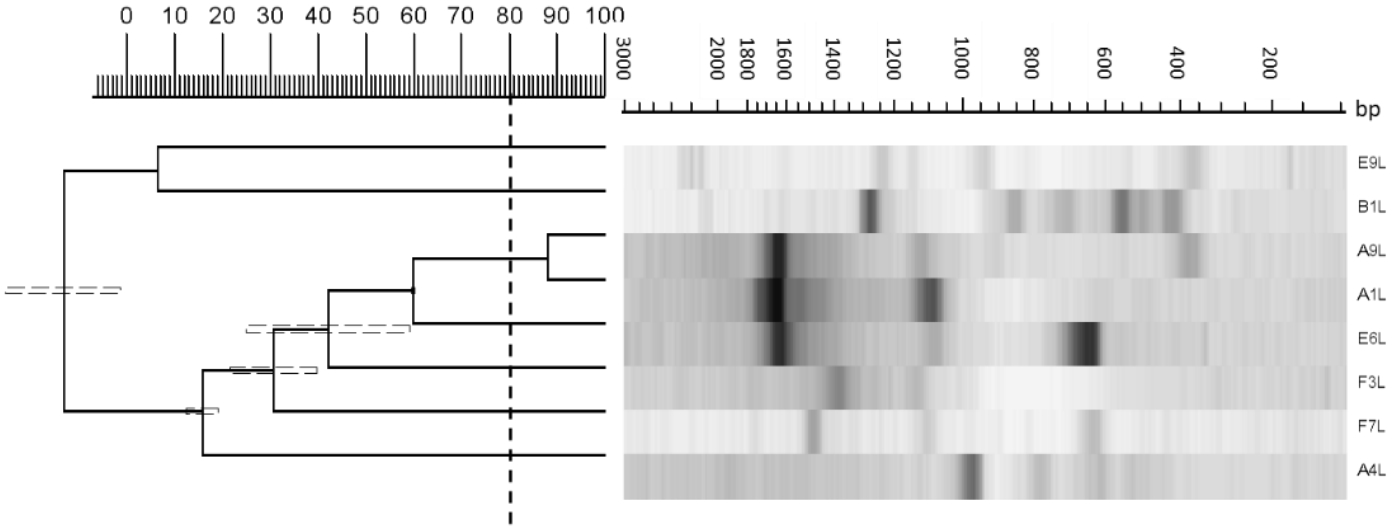

Figure 4. Dendrogram drawn by UPGMA of correlation value of normalized $(GTG)_5$-PCR patterns from lactobacilli obtained with primer $(GTG)_5$. Each pattern is identified by the codification of the strain. The coefficient of similarity (80%) is materialised by a vertical dotted line.

Table 2. Means and standard deviations (3 repetitions) of pH, titratable acidity (TTA) and organic acid rates, after 72 h of fermentation in carrot-like medium. Final pH refers to the value at the end of the fermented step. Other data are expressed as the difference between 72 h and the initial time. TTA is expressed in µL and lactic acid in mg/L; nd: not detected.

Strain	Final pH	pH	TTA	L-Lactic acid	D-lactic acid
L. paracasei A1L	3.57 ± 0.13	-1.67 ± 0.23	620.0 ± 272.2	713.2 ± 254.7	19.22 ± 14.68
L. paracasei F7L	3.53 ± 0.07	-1.80 ± 0.08	740.0 ± 132.3	873.8 ± 148.5	n.d
L. harbinensis A9L	4.10 ± 0.02	-1.22 ± 0.03	193.3 ± 37.8	435.3 ± 35.6	n.d
L. harbinensis E6L	3.89 ± 0.01	-1.40 ± 0.03	266.7 ± 41.6	466.5 ± 63.0	n.d

and 3.89 ± 0.01. TTA differences were higher for *L. paracasei* strains, with NaOH volumes equal to 620.0 ± 272.2 and 740.0 ± 132.3 µL. According to TTA changes, the L-lactic acid quantities measured were higher with *L. paracasei* strains in comparison with *L. harbinensis* strains, with values at the end of fermentation of 713.2 ± 254.7 and 873.8 ± 148.5 mg/L. *L. paracasei* A1L strain produced 19.22 ± 14.68 mg/L of D-lactic acid, unlike other strains, for which D-lactic acid was not detected. Acetic acid was never measured whatever the strain considered. pH, TTA and lactic acid concentrations were analysed by analysis of variance (ANOVA). *L. paracasei* and *L. harbinensis* appeared significantly different ($p<0.001$).

The two strains of *L. paracasei* were able to produce higher lactic acid concentrations, - and consequently they strongly acidified the medium - than *L. harbinensis*. However, within each species, the two strains could not be separated from each other ($p>0.05$).

DISCUSSION

The present study focused on the valorisation of carrot pulp wastes and the selection of LAB strains for their ability to produce L-lactic acid. Lactic acid being used, for instance, in bioplastic production, the optimization of this processing represents an industrial challenge. Among the dominant microflora of the fermented vegetables, LAB are frequently found(Gänzle, 2015). In order to select a strain of technological interest, two culture media were used. The MRS medium was considered as a control for the fermentation. A sterile carrot-like medium was developed to test the microorganisms in an environment as close as possible to the vegetable substrate. We isolated an interesting lactic acid bacteria from fermented carrot pulps, which was further identified as *L. paracasei* A1L. LAB were isolated from samples of fermented carrot pulps submitted to two different temperatures, 24°C or 37°C (Godard et al., 2018). Samples were picked at

Table 3. *Lactobacillus* species identified in the literature during vegetable fermentation. Groups were defined according to Salvetti et al. (2012) and Gu et al. (2013).

***Lactobacillus* group**	**Species of this study**	**Fermented vegetable**	**References**
L. buchneri	*L. parabuchneri*	Wet wheat, cabbage, lettuce	(Olstorpe et al., 2010; Yang et al., 2010a)
	L. sunkii	Pickle	(Watanabe et al., 2009)
	L. otakiensis	Pickle	(Watanabe et al., 2009)
L. perolens	*L. perolens*	Wetwheat	(Olstorpe et al., 2010)
	L. harbinensis	Chinesesauerkraut	(Miyamoto et al., 2005; Sagdic et al., 2014)
L. casei	*L. rhamnosus*	Chinesesauerkraut, cabbage, lettuce	(Yang et al., 2010a; Yang et al., 2010b)
L. delbrueckii	*L. delbrueckii*	Cabbage, lettuce	(Yang et al., 2010a)
L. coryniformis	*L. coryniformis*	Cabbage, lettuce	(Yang et al., 2010a)
	L. bifermentans	Kimchi, cabbage, lettuce	(Jhon and Lee, 2003; Yang et al., 2010a)
L. vaccinostercus	*L. nenjiangensis*	Pickle	(Gu et al., 2013)
L. koreensis	*L. koreensis*	Kimchi	(Bui et al., 2011)
L. plantarum	*L. plantarum*	Wet wheat, leek, Romanian vegetable, carrots, sauerkraut, pickle	(Gänzle, 2015; Olstorpe et al., 2010; Wouters et al., 2013; Wouters et al., 2013b)
	L. mudanjiangensis	Chinese pickle	(Gu et al., 2013)

several stages during the nine days of the fermentation step. Cultural-dependant approach was chosen to allow us the isolation of viable and cultivable LAB from Petri dishes.

Whatever the sampling stage, LAB ranged between 7.57 and 9.07 log CFU/mL. Using cultural methods, Wouters et al. (2013b) investigated the microbial diversity during the fermentation of cauliflower and vegetable mix (tomatoes, carrots and celery). At the beginning of the fermentation step, the authors counted lower LAB rates - between 1 and 2 log CFU/mL - compared with our results obtained in fermented carrot pulps. However, after 1 to 2 months, the levels were similar with ours, values ranging between 5.3 and 11.4 log CFU/mL following the substrate. Among the 253 isolates collected, the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Weissella* were identified using the (GTG)5-PCR method. In our study, we only found the two genera *Lactobacillus* and *Leuconostoc*. This association is frequently noticed when vegetables are fermented. It is assumed that the heterofermentative metabolism of *Leuconostoc* linked with their ability to produce exopolysaccharides could explain their aptitude to survive in harsh conditions (Demarigny, 2012). In a vegetable environment, heterofermentative cocci, and among them *Leuconostoc*, produce gas from carbohydrate sources, generating advantageous growth conditions for lactobacilli (Yang et al., 2010a). The 40 strains identified from carrot pulps were shared into nine *Lactobacillus* groups, seven being already known for their implication in vegetable fermentations. Among them, *L. plantarum* has been described for long time as very adaptable to drastic conditions. This microbe is found in many food products such as pickles, sauerkraut or olives (Gänzle, 2015). This bacterium owns many genes able to degrade various carbon sources (Kleerebezem et al., 2003). Moreover, *L. plantarum* is rather resistant to the low pH (below 4) that are frequently reached during vegetable fermentation (Todorov and Franco, 2010). In our case, *L. plantarum* proved to be less frequent than awaited. The specific physico-chemical characteristics of the carrots could explain this observation.

In the fermented carrot pulps, the main species identified were *L. paracasei*, *L. nenjiangensis* and *L. harbinensis*. As stated before in the literature, they were found in different food products, Chinese sauerkraut for example (Table3). We also identified some other species of *Lactobacillus* and *Leuconostoc*, but at low frequencies. Among them, *L. coryniformis, L. bifermentans, L. parabuchneri, L. rhamnosus* and *L. delbrueckii* were also isolated from the surface of cabbage and lettuce leaves (Yang et al., 2010a). As indicated in the result part, nine of the strains out of forty exhibited significant acidifying capacities on modified MRS broth; L-lactic acid rates ranged between 67 and 960 mg/L depending on the strain. The modified MRS medium did not contain dipotassium phosphate. It was not buffered to allow the pH and the titratable acidity to vary during the culture. Moon et al.(2012) and Zhang and Vadlani.(2015)used a similar approach to screen *Lactobacillus* strains submitted to different conditions. This allowed them to test the metabolic activity of their microorganisms in controlled and growth enhancing model media, rich in glucose and available nitrogen compounds.

Some of our strains were homofermentative (*L. mudajiangensis* and *L. porcinae*) and the other facultatively heterofermentative (*L. paracasei*, *L. nenjiangensis*, *L. harbinensis*, *L. coryniformis* and *L. kimchii*). On the basis of the results obtained with the MRS medium, cultures in carrot-like medium were carried out in order to mimic what could be the microbial development in the carrot pulps. Results confirmed the trends observed on MRS medium. *L. paracasei* and *L. harbinensis* mainly produced L-lactic acid (>93% of the total amount of lactic acid). If the metabolic characteristics of *L. paracasei* are now well documented in some products (cheeses), the role of these two species in fermented vegetable products is still largely unknown. For example, *L. paracasei* can be added during cheese making for its probiotic abilities (Buriti et al., 2007). This bacterium is able to survive to acid pH (3) and to bile salts (0.3%), which are conditions as harsh as those found in the digestive tract (Kask et al., 2003).

In modified MRS broth, the lactic acid production of *L. harbinensis* was 10% higher than those obtained with *L. paracasei*; whereas in carrot-like medium, *L. paracasei* produced 1.6 times more of lactic acid. The lactic acid concentrations were respectively 14 and 24 times lower in carrot-like medium compared to MRS medium, whatever the species considered. In all cases, the D-lactic acid rate was very low, no more than 2 to 6% of the total acid produced; acetic acid was never detected. These facultative heterofermentative lactobacilli seemed therefore to favour the homofermentative pathway. Such a metabolic choice is largely dependent on the physico-chemical and nutritional parameters of the environment. For instance, Prückler et al. (2015) reported that the two heterofermentative *L. plantarum* and *L. pentosus* cultured on wheat bran – a substrate which contains high concentrations of available glucose -, only produced lactic acid (41.75 mg/g). Acetic acid was not detected as well. In the meantime, *L. acidophilus* and *L. delbrueckii* (strictly homofermentative and thermophilic bacteria) were unable to release lactic acid under the given conditions. However, these experiments were made at 30°C. This temperature being lower than the optimal growth temperature of these two last microorganisms, their low activity seemed logical. As observed in the course of our experiments, *L. plantarum* and *L. pentosus* did not produced acetic acid. Moon et al.(2012)used the strain *L. paracasei* subsp. *paracasei* CHB2121 to produce L-lactic acid in MRS broth. After 21 h of culture, the lactic acid concentration in the medium was close to 95 g/L for a yield of 93%. In presence of glucose as the only carbon source, the *L. paracasei* metabolism led to the exclusive production of lactic acid.

Based on the $(GTG)_5$-PCR profiles, we can argue that the strains we isolated were clearly distinct. This assertion was confirmed by the analysis of some of their respective abilities; in particular, the use of specific carbohydrates. For example, *L. paracasei* A1L was able to use starch. This polymer is frequently found inside plant cells. The concentration of starch in raw carrots is close to 0.8 g/100 g. This aptitude can then give a putative selective advantage to the bacteria(Anses, 2017). The carrot pulp used to make the carrot-like medium was sterilized to avoid any competition with the adventitious microorganisms present in the raw pulp. In a later work, we will test the settlement of our strains under the pressure of the other microbes. It is so important as native bacteria are generally known to be well adapted to their environment. Wouters et al. (2013a) investigated the influence of the addition of a selected *L. plantarum* strain during the fermentation of vegetable mix (carrot, tomato and cauliflower). Compared with the control, a rapid acidification rate of the inoculated samples was observed.

We are also aware of the strong variability of the carrot pulp characteristics - depending on the carrot species, harvesting conditions, season or adventitious microbial flora. Consequently, L-lactic acid concentrations depend on the physico-chemical environment of the matrix,which is likely to impair the results; this will be later assessed. The measures we made on the raw carrots were usual – pH 5.2, Aw 0.99. This is frequently found in fresh carrots, the pH ranging between 4.9 to 5.2. Such a pH value can be considered as favourable to support the growth of most of the *Lactobacillus* strains. It is assumed that, depending on species and strains, lactobacilli can cope with pH as low as 3. If we consider Aw, a level of 0.99 favours the growth of any microbes (Jay et al., 2005). The physico-chemical conditions of the pulps seemed therefore adapted to their re-use as fermentation substrate. This work brought evidences that the addition of lactobacilli could appear a good option to obtain bio-lactic acid. From now on, it is necessary to optimize the culture conditions to maximize the lactic acid production.

Conclusion

In this study, we showed that *L. paracasei* A1L was able to produce L-lactic acid in a carrot substrate. However, at that time, the yields are still too low to allow its transfer at the industrial scale. Consequently, experiments have to be made to optimize the lactic acid production. The first optimization step involves the extrinsic and intrinsic factor adjustment, mainly the temperature and the pH; but also the sugar or nitrogen compound concentrations. The second step will focus on inhibiting factors that could impair lactic acid production; and that would be specific to carrot pulp. In the context of industrial processes, it could be interesting to use co-cultures with two *L. paracasei* strains to avoid phage infection or with one *Lactobacillus* and one *Leuconostoc*. The compatibility of the two strains and their ability to cooperate in the same ecosystem will

have to be tested. To conclude, scale-up will have to be taken into account to obtain sufficient yields of lactic acid.

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