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Evolution of carrot pulps during spontaneous fermentation

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ABSTRACT

The spontaneous fermentation of carrot pulps was followed under different conditions during nine consecutive days. Trials were carried out to obtain interesting compounds: organic acids and volatile molecules. Factors were tested with a two-level full factorial design: temperature (24°C/37°C), grinding (with/without), addition of water (0%/50% w/w) and refreshment of the fermentation medium (without/after 3 or 7 days of incubation). During the course of the fermentations, different parameters were measured: microbial gross composition and diversity (REP-PCR), pH, lactic and acetic acids, ethanol, phenolic and volatiles compounds (SPME-GC-MS). Whatever the culture conditions tested, the pulp ecosystem remained quantitatively stable from the beginning to the end of the culture step. It was dominated by lactic acid bacteria (LAB), followed by Enterococci, yeasts, moulds and acetic acid bacteria (AAB); *Enterobacteriaceae*, *Bacillus* and *Pseudomonas* were found at low levels. However, the composition of each population changed. As a consequence, the LAB proved to be the most influencing population on the production of ethanol, acetic acid and lactic acid. 2-Butanol was also detected in the fermented pulps; but no relation was found with the microorganisms looked for. Among the factors tested, the rise in temperature led to a decrease in the production of acetic acid, ethanol and D-lactic acid.

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INTRODUCTION

Throughout the world, one-third of alimentary products go unused. This translates into around 1.6 billion tonnes of waste and \$936 billion of lost food. Vegetables, fruits and cereals represent between 15 and 25% of total waste (FAO, 2013). Currently, these wastes are mainly recycled by bioconversion, animal feed or energy production (such as incineration). Such by-products are for the most part removed during the harvesting or food making phases and domestic waste (Abu Yazid et al., 2017).

In the literature, the use of vegetable wastes has mostly

focused on the production of biomolecules, including organic acids – citric, lactic and gallic acids –, bioethanol, aroma compounds, biopesticides, enzymes and biosurfactants. This is partly explained by the increasing economic interest in these substances. For example, the market of organic acids was estimated at 6.55 billion USD in 2015. Wastes are transformed by the action of microbes to obtain these interesting compounds. Lactic acid, a product of the lactic acid bacteria (LAB) metabolism, has been widely reviewed in the literature (Eş et al., 2018). Citric acid is used in pharmaceutical, cosmetic and food industries. For example, a yield of 29 g of citric acid per kilogram of dry matter was obtained after the fermentation of carrot wastes by *Aspergillus*

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niger. It is low compared with the yield obtained on apple pomaces with the same mould: between 124 and 883 g/kg of product, depending on the fermentation conditions (Vandenberghé et al., 2018). Biosurfactants are molecules able to modify the surface properties of other substances. For instance, they can be used to decontaminate polluted environments by hydrocarbon, or as antimicrobial agents (Youssef et al., 2004).

To ferment a substrate, some parameters have to be checked carefully to maximise the production of the desired molecules: pH, temperature, nutrient composition, microbial composition, water activity, aeration, pre-treatment and particle size (Abu Yazid et al., 2017; Pandey et al., 2000). In this latter case for instance, whereas tiny particles increase the exchange surface, large particles tend to favour aeration but decrease the adherence surface for microorganisms. The water activity ratio needed for growth is different for bacteria - between 0.8 and 0.999 - and fungi - above 0.6. Linked to the influence of the particle size, moisture can impair oxygen diffusion inside the medium. Temperature is a key parameter as it impacts the microbial growth rate, which can differ slightly from the maximum rate of molecule production.

Recently, it has been shown that lactic acid production was influenced by the cross effect of temperature and pH. This was observed in food waste spontaneous fermentations (rice, vegetables and meat) initiated by the addition of the LAB: *Lactobacillus* and *Weissella*. The optimisation of acid production requires a temperature close to 37°C and a pH of 6. This optimum was confirmed by the construction of a quadratic mathematical model (Tang et al., 2016).

During the production of grated carrots, about 25-35% of the raw material is lost in the form of carrot pulps (peels and fallen leaves including soil particles). The biochemical composition of carrot wastes is particularly effective in producing various compounds: acetic and lactic acids, ethanol and lipopeptides. Carrots contain 1-1.2% - fat, 32-64.3% - carbohydrates, 7.2-8.6% - crude protein, 4-12.3% - hemicellulose and 43.3% - phenolic compounds (Banerjee et al., 2017; Wadhwa and Bakshi, 2013). Different articles have been published on the use of this substrate as a fermentative matrix.

In this work, we used carrot pulps to obtain valuable compounds after a fermentation step. Unlike Di Cagno et al. (2008), we only used the adventitious microflora present in the pulps themselves to stimulate fermentation; no starters were added. We tried to favour compound production by modifying the physico-chemical parameters of the environment: temperature, water addition, grinding and refreshment.

MATERIALS AND METHODS

Four parameters were selected to test their influence on

microbial developments: temperature, grinding of the pulps, addition of water and refreshment of the fermentation medium. In order to identify the most influent technological parameters among the four selected, a two-level full factorial design was built (2^4 , 16 experiments plus three trials in the centre of the domain). For each factor, a low and a high level were determined depending on the technological restrictions (Table 1). The central point was triplicated to estimate the variability of the experimental results.

Vegetable pulp was composed of carrot peels. The products were transported from a plant producing grated carrot. Pulps were kept at 4°C from sampling to analysis. The travel and the storage did not exceed 24 h. Fermentations were conducted in nineteen 500 mL Erlenmeyer flasks (identified thereafter by the codes "S1" to "S19"). Three hundred and twenty grams of raw pulp were added to each flask, with water if required by the experimental design. The fermentation lasted for 9 days (identified thereafter by the letters "D0" to "D9"). Where specified, samples were ground using a blender for 1 min (Ultra Compact Plunge Mixer, SEB®, France). When refreshment was required, the supernatant was completely extracted and replaced with sterile water (w/w). The total solid content of the fermentation material was shown in Table 2.

Sampling depended on the type of analysis performed: on days 0, 1, 2, 3, 7 and 9 for acetic acid, D- and L-lactic acid, ethanol, phenolic compounds, pH and total titratable acidity (TTA); on days 0 and 9 for quantitative and qualitative microbial analyses.

Sampling for microbial and chemical analyses

During the fermentation, 8 g of carrot pulp were suspended in 72 g of sterile physiological water (0.9%) and homogenised with a stomacher apparatus (Samasher®, Biomérieux, France) for 1 min, at fast speed. A part of the supernatant of the resulting slurry was frozen with glycerol (15%, v/v) at -80°C awaiting microbial analysis. The rest of the supernatant was stored at -20°C for chemical analysis.

Microbial analyses

The following microbial groups were determined: LAB on de Man-Rogosa-Sharpe agar (MRS) agar supplemented with pimaricin (0.1 g/L, Sacco System, Italy) and nalidixic acid (0.04 g/L, Merck KGaA, Germany), and M17 agar supplemented with pimaricin (0.1 g/L), both media at 30°C for 48–72 h; *Pseudomonas* on cephalosporin-fucidin-cetrimide (CFC) agar incubated at 22°C for 72 h; *Enterobacteriaceae* on violet-red-bile-glucose (VRBG) agar at 37°C for 24 h; and Enterococci on bile-esculine-

Table 1. Experimental matrix of two-level full factorial design (2^4), for spontaneous fermentation of carrot pulp with the four variables tested. Central point was triplicated.

| Sample (S) | Grinding | Temperature (°C) | Percentage of added water (w/w) | Day of refresh (D) |
|------------|----------|------------------|---------------------------------|--------------------|
| S1 | Without | 24 | 0 | D0 |
| S2 | With | 24 | 0 | D0 |
| S3 | Without | 37 | 0 | D0 |
| S4 | With | 37 | 0 | D0 |
| S5 | Without | 24 | 50 | D0 |
| S6 | With | 24 | 50 | D0 |
| S7 | Without | 37 | 50 | D0 |
| S8 | With | 37 | 50 | D0 |
| S9 | Without | 24 | 0 | D3 and D7 |
| S10 | With | 24 | 0 | D3 and D7 |
| S11 | Without | 37 | 0 | D3 and D7 |
| S12 | With | 37 | 0 | D3 and D7 |
| S13 | Without | 24 | 50 | D3 and D7 |
| S14 | With | 24 | 50 | D3 and D7 |
| S15 | Without | 37 | 50 | D3 and D7 |
| S16 | With | 37 | 50 | D3 and D7 |
| S17 | Without | 30.5 | 25 | D3 |
| S18 | Without | 30.5 | 25 | D3 |
| S19 | Without | 30.5 | 25 | D3 |

Table 2. Total solid content of fermentation material in percentage of dry matter. Mean±SD values for three repetitions of each condition of fermentation.

| Percentage of dry matter | | With grinding | Without grinding |
|---------------------------------|------|---------------|------------------|
| | 0 % | 4.87±0.18 | 6.32±0.64 |
| Percentage of added water (w/w) | 25 % | / | 4.32±0.45 |
| | 50 % | 2.69±0.11 | 3.62±0.39 |

azide agar at 37°C for 24–48 h. For the enumeration of acetic acid bacteria (AAB), the composition of the medium used, for one litre, was as follows: bacteriological peptone (10 g, Oxoid), yeast extract (3 g), lactic acid (6.3 g, Prolabo, France), ethanol (5 g), acetic acid (3 g, AppliChem GmbH, Germany), glucose (1 g), sorbitol (1 g), mannitol (1 g), potassium hydrogen phosphate (1 g), sodium deoxycholate (0.1 g), magnesium sulphate heptahydrate (20 mg), bromocresol purple (30 mg), pimaricin (0.1 g) and agar (18 g) (Sigma), according to Papalexandratou et al. (2013). The pH of the medium was adjusted to pH 4.5. AAB plates were incubated at 30°C for 10 days. *Bacillus* were counted on plate-count-agar (PCA) agar supplemented with 2,3,5-Triphenyltetrazolium chloride at 0.1 g/L (Merck KGaA). Ten milliliters of the tested solution were first heated at 80°C for 10 min before being poured on the surface of the medium (1 mL/plate). Yeasts and moulds were

sought for on chloramphenicol-glucose-agar (CGA) agar, at 25°C for 48 h.

With the exception of MRS agar, all the media were incubated in aerobic conditions. Except when specifically indicated, all the media were purchased from Biokar diagnostics (France).

“Scrapings” and bacterial DNA extraction

When the number of microbial colonies was counted at between 50 and 500, 2 mL of physiological water were poured on the surface of the medium and the colonies were scraped off with a rake. This suspension was then centrifuged at 20,000 $\times g$ for 3 min. Cells were washed with 1 mL of physiological water; this procedure was repeated twice.

For DNA extraction, the pre-lyse step was adapted to

Nucleospin® Tissue kit (Macherey-Nagel, Germany). Twenty microliters of lysozyme (10% w/v, Lysozyme from chicken egg white, Sigma, Germany) was added to the lysis buffer and incubated at 37°C for 30 min. Twenty microliters of RNase (1 g/L, Ribonuclease A, Sigma) were then added and the solution was kept at 37°C for 30 min. Finally, 30 µL of proteinase K (1 g/L) and 20 µL of sodium dodecyl sulfate (SDS, 0.1 g/L) were added. The mix was first kept at 37°C for one night and then at 64°C for 30 min. For encapsulated bacteria DNA extraction, cetyltrimethyl ammonium bromide (CTAB, Sigma; 0.1 M Tris-HCL pH8, 1.4 M NaCl, 20 mM EDTA and 0.05 M) was added at the end of the lyse step (Mertens et al., 2014; Moreira et al., 2010; Yalçınkaya et al., 2017).

REP-PCR amplification and profile analysis

Bacterial DNA was amplified as described by Dalmasso et al. (2008). REP-PCR was performed using Rep1 (5'-IIICGNCGNCATCNGGC-3') and Rep2 (5'-NCGNCTTATCNGGCCTAC-3') primers. DNA fragments were separated on agarose gel electrophoresis (1% in EDTA-buffer), after 4 h of migration at 80 mV. The 123 bp DNA Ladder (Merck KGaA) was used to determine band size. The band patterns were normalised and processed using GelCompar 3.1 software (Applied Maths, Kortrijk, Belgium). Diversity indices were calculated according to the Shannon and Weaver formula:

$$H = \sum P_i \log P_i$$

Where, P_i is the number of profiles with 82% of similarity/total number of profiles.

SPME/GC-MS analysis

Volatile compounds were analysed using the Solid-Phase MicroExtraction Gas-Chromatography technique (SPME/GC, 6890 Series GC System, Hewlett Packard), coupled with Mass-Spectrometry (MS, 5973 Mass Selective Detector, Hewlett Packard). Ten millilitres of supernatant were placed in a vial and heated at 60°C for 5 min. SPME-fiber was conditioned at 270°C for 30 min. Compounds were absorbed onto this fiber (SPME Fiber Assembly DVB/CAR/PDMS, Supelco, Sigma) at 60°C for 45 min. They were then desorbed and separated with a gradient of temperature according to Aggelopoulos et al. (2014). Helium was used as vector gas with a flow rate at 1 mL/min. Molecules were separated through a BPX 5 capillary column, 30 m × 0.25 mm i.d. × 0.25 µm, 5% diphenyl and 95% dimethylpolysiloxane (Phenomenex, Le Pecq, France). They were identified using Chemstation software by spectra comparison with NIST

05 and NIST 98 libraries.

Physicochemical parameters

pH of the supernatants was determined using a pH meter (HI 2211, pH/ORP Meter, Hanna instruments). Total phenolic contents were measured according to the methodology proposed by Kaprasob et al. (2017). Concentrations of citric, acetic and lactic acids, and ethanol were measured with enzymatic kits (R-Biopharm, Germany).

Statistical analyses

The influence of the parameters (temperature, grinding...) on the different factors we followed (organic acids, volatile molecules...) were tested according to a multiple linear regression (MLR) procedure using the Modde V10 software (Sartorius Stedim Data Analytics AB Malmö, Sweden). The data gathered throughout the experiment (Shannon and Weaver diversity indices, levels of microbial populations, physicochemical measures), were analysed using the XLSTAT software.

RESULTS AND DISCUSSION

Microbial evolution during fermentation

As expected, the variations between the levels of the different populations were very slight on D0 (Figure 1A). The raw pulp was homogeneous, the standard deviation being lower than 0.5 log CFU/mL, irrespective of the microbial community considered. This observation was confirmed by Ascending Hierarchical Classification analysis which allowed us to highlight three groups of microorganisms (data not shown). LAB were preponderant in carrot pulps, their cell density ranging from 7.44 to 8.69 log CFU/mL. In raw vegetables, LAB are generally counted at lower levels - between 2 and 4 log CFU/mL (Di Cagno et al., 2013; Paramithiotis et al., 2010; Wouters et al., 2013b). This implies that the pulps collected in the course of this experiment had already begun to ferment during the storage of the pulps in the plant. Enterococci, AAB, and yeasts and moulds were enumerated at sub-dominant levels - from 5.00 to 7.22 log CFU/mL. *Enterobacteriaceae*, *Bacillus* and *Pseudomonas* did not exceed 5.00 log CFU/mL in the carrot pulp ecosystem. The microbial diversity of raw vegetables and fruits has frequently been described. The authors observed dominant and constant populations generally composed of yeasts and fungi (2 to 6 log CFU/mL), *Enterobacteriaceae* (4 to 5 log CFU/mL) and *Pseudomonas* (no greater than 5 log CFU/mL). In raw

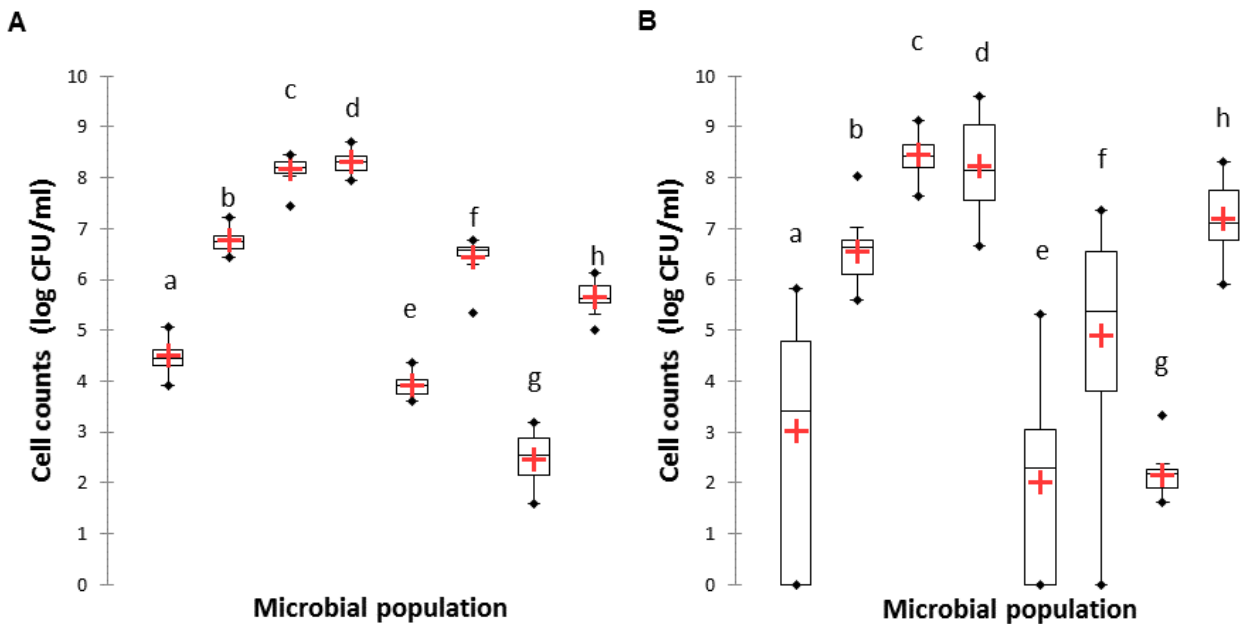


Figure 1. Levels of Enterobacteriaceae: **a**, Yeasts and moulds; **b**, LAB (rod shape); **c**, LAB (coccus shape); **d**, *Pseudomonas*; **e**, Enterococci; **f**, *Bacillus*; **g**, acetic acid bacteria; **h**, obtained from the 19 carrot pulp samples of the design (S1 to S19), at D0 (A) and D9 (B). Cell numbers are expressed in log CFU/mL.

vegetables, LAB were not so numerous, their concentration varying between 2 and 4 log CFU/mL. In our study, the microbial ecosystem composition was similar, except for Enterococci, which reached 5.3 log UFC/mL and rod shape and coccus shape LAB - 7.4 log CFU/mL.

After 9 days of fermentation, the development of the microbial populations under observation did not change significantly (Figure 1B). Some slight differences were noticed but the ratios between the populations remained roughly identical. LAB were still present at high levels - between 7.5 and 9.5 log CFU/mL. These results differ from the former data reported in the literature. Di Cagno et al. (2008) for instance investigated the capacity of LAB to develop in a carrot substrate, either after starter addition or based on the adventitious microflora. After 8 days of culture, they observed that the LAB level of the starter samples was close to 9.38 log CFU/mL whereas in the non-starter medium, this level was 1 000 to 10 000 times lower. The medium used by these authors was different from ours, which can explain these differences. For instance, the wastes they used (carrot juice, French beans or marrows) were first heated at 121°C for 10 min before fermentation.

Between D0 and D9, the extent of each population level varied greatly, especially for *Pseudomonas*, *Enterobacteriaceae* and Enterococci, which all decreased. In the case of the first two populations, a similar occurrence has also been observed by other

authors; for example, in a leek fermented medium (Wouters et al., 2013a). We can suppose that dominant LAB exerted a negative influence on the development of the other microbes. The level of AAB increased – the difference between D0 and D9 ranged from 0.3 to 2.35 log CFU/mL following the sample. Yeasts and moulds decreased slightly over the 9 days of fermentation (-0.5 log CFU/mL). These microorganisms have frequently been enumerated in vegetable raw materials (Di Cagno et al., 2013). The development of *Pseudomonas*, *Enterobacteriaceae* and yeasts and moulds appeared to correlate (Principal Component Analysis, results not shown). This result concurs with the observations made by Di Cagno et al. (2008).

REP-PCR profiles were performed by scraping off all the colonies obtained on the petri dishes. As such, the profiles obtained can be considered as an image of the dominant and sub-dominant viable and cultivable cells present in the pulps at a given step. An example of a profile is shown on Figure 2 for the rod shape LAB at D0 (Figure 2A) and D9 (Figure 2B). But the same procedure was carried out on all the media at the beginning and at the end of the fermentation step. Based on a coefficient of similarity of 82%, it was possible to calculate the diversity indexes for each bacterial community (Table 3). Three groups of behaviour were observed. For coccus shape LAB, Enterococci and *Bacillus*, the diversity indexes did not change from D0 to D9. They were very high for LAB and *Bacillus* flora, - respectively, $H_{D0} = 1.03$

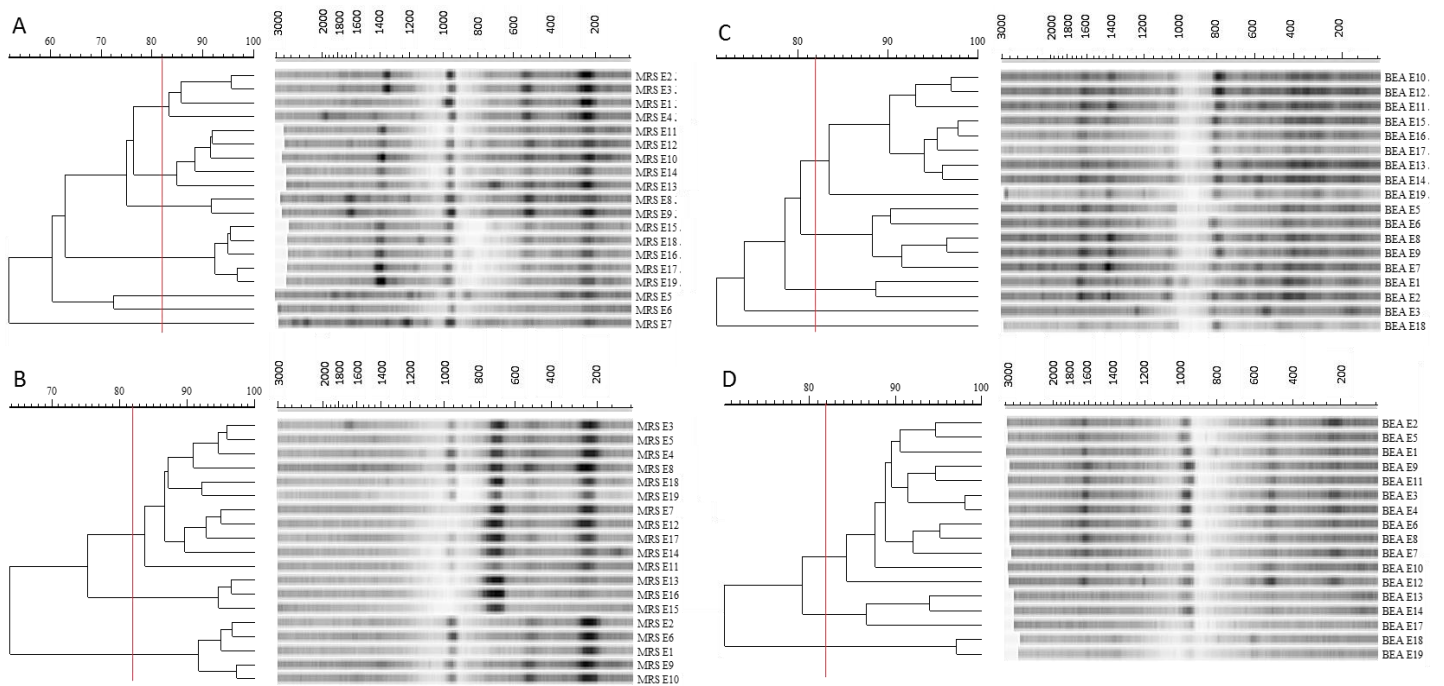


Figure 2. REP-PCR profiles of carrot pulp samples, for rod shape LAB (rod shape, A and B) and Enterococci (C and D), at D0 and D9, respectively. The coefficient of similarity (82%) is materialised by a vertical red line.

Table 3. Shannon and Weaver diversity indexes; they were calculated for all bacterial communities, at D0 and D9 of fermentation, for the 19 fermentation samples of carrot pulps (S1 to S19).

| Medium | Diversity index H |
|------------------------------|-------------------|
| LAB (coccus shape) D0 | 1.03 |
| LAB (coccus shape) D9 | 1.01 |
| <i>Pseudomonas</i> D0 | 0.56 |
| <i>Pseudomonas</i> D9 | 0.32 |
| Enterococci D0 | 0.33 |
| Enterococci D9 | 0.35 |
| AAB D0 | 0.68 |
| AAB D9 | 0.26 |
| LAB (rod shape) D0 | 0.75 |
| LAB (rod shape) D9 | 0.42 |
| <i>Bacillus</i> D0 | 0.98 |
| <i>Bacillus</i> D9 | 0.94 |
| <i>Enterobacteriaceae</i> D0 | 0.23 |
| <i>Enterobacteriaceae</i> D9 | 0.64 |

and 0.98 and $H_{D9} = 1.01$ and 0.94. However, as shown on Figure 2, the profiles obtained at D9 had changed compared with D0. While some bands were still present on D0 and D9 (250 pb and 900 pb), others had disappeared (1300 pb) or appeared (700 pb). We can then infer from these observations, that although the

diversity remained stable, the LAB ecosystem had been partially renewed. According to Di Cagno et al. (2008), in vegetables and particularly in carrots, the species *Leuconostoc mesenteroides* subsp. *mesenteroides* is generally dominant during spontaneous fermentation. Wouters et al. (2013a) identified other LAB

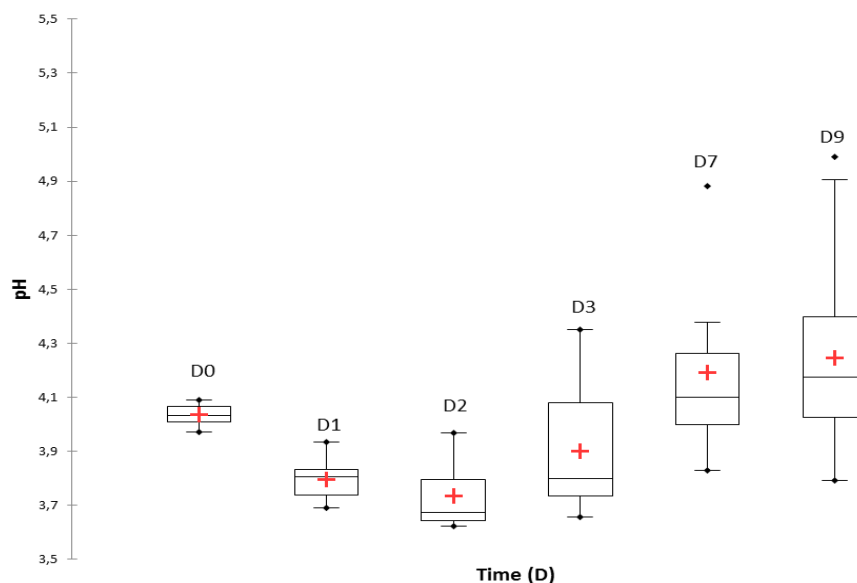


Figure 3. Change in pH during the fermentation of the 19 carrot pulp samples of the design (S1 to S19), from D0 at D9.

species during leek fermentation, *Lactobacillus sakei*, *Lb. plantarum*, *Lb. brevis*, *Weissella soli* and *Leuconostoc gasicomitatum*. In both cases, the samples contained a similar level of LAB. *Lb. plantarum* is also commonly found in products resulting from spontaneous fermentations (Demarigny, 2012). For Enterococci (Figure 2C and 2D), except for one common band, the profiles obtained at D0 and D9 were different, meaning that the ecosystems of these two populations had almost completely changed as a consequence of the fermentation step. The same observation can be made for *Bacillus*.

The second group of profiles included the rod shape LAB, *Pseudomonas* and AAB populations. The diversity indexes decreased strongly concomitantly with a modification of the profiles. The last group included the *Enterobacteriaceae* population, which seemed to adapt to the fermentation conditions. Indeed, while the counts showed a decrease in the number of cells, diversity increased. It is noticeable that irrespective of the index evolution and whatever conditions were applied, the final profiles obtained at D9 were identical, especially for the dominant populations. It shows that the dynamic of the microbial communities, in the range defined by the design we followed, was remarkably stable. Similar results have already been obtained on other substrates - vegetable wastes and cocoa bean vessels - by Lefeber et al. (2011), Liang et al. (2016) and Wouters et al. (2013).

The presence of common bands on different profiles, for instance at around 1 000 pb for rod shape LAB and Enterococci at D9, allows us to think that it could correspond to the same population. Indeed, the media

used are specifically selected to search for a genus we expect to encounter. It has been well known for a long time that petri dishes give a crude image of the population being looked for (Gemelas et al., 2013; Verdier-Metz et al., 2012). The sequencing of these specific bands could be of further interest to identify in detail the populations concerned, and in doing so we could evaluate the effectiveness of the methodology followed here.

Physico-chemical parameters

Generally speaking, the pH of the samples decreased from D0 to D2, - from 4.03 to 3.73. Thereafter, the pH measures increased widely over the course of the fermentation period to reach 4.2 ± 0.3 at the end of the fermentation step (Figure 3). It means that the samples evolved differently as the consequence of the parameters applied. The temperature in particular had a strong impact. This is the case for S3, S4 and S11 samples which were incubated at 37°C. Their final pH was respectively equal to 4.99, 4.91 and 4.51, 0.5 to 0.8 unit above the mean of the other samples. Di Cagno et al. (2008) observed the same variations on carrot media amended with an autochthonous LAB starter.

Citric acid, a compound frequently found in vegetal cells, was never detected in the supernatant. We can suppose that citric acid was either consumed or was not available for microbes and remained in the solid matters of the samples. Acetic acid concentration was highest at D3, with a mean of 319 mg/L. Thereafter, the rate

Table 4. Flavours associated with molecules identified during carrot pulp fermentation (Acree and Arn, 2012; Kim et al., 2015).

| Flavour | Compounds (PubChem name) |
|-----------|--|
| Sweet | Ethanol, caryophyllene |
| Vegetable | Octanal, caryophyllene (moss, earth), geranylacetone (mouldy) |
| Fruity | Ethyl acetate (pineapple), isobutanol (wine), ethyl butyrate (apple), ethyl valerate, heptanal (citrus), ethyl caproate, octanal (citrus), O/P/M-cymene (citrus), ethyl octanoate, geranylacetone (Tomato) |
| Others | 2-Butanol (solvent), isobutanol (solvent), heptanal (fat, rancid), ethyl caproate (dairy produce), octanal (fat), ethyl octanoate (dairy produce) |

decreased to stabilise at 190 mg/L after nine days. D and L-Lactic acid were as high as 254 and 177 mg/L respectively, at D2. The presence of these two acids seemed to correlate with the evolution of pH, even if no significant relation was observed. The presence of lactic and acetic acids, and ethanol was also reported during pulp carrot fermentation by Wouters et al. (2013a). But unlike what these authors found, in our case, acetic acid concentration was higher than that of lactic acid after 9 days of incubation. The rate of phenolic compounds remained stable during the whole fermentation step, at around 4.57 g gallic acid equivalent/L.

Evolution of the volatile compound composition from the beginning to the end of the fermentation step

Among the 208 chemical compounds obtained after static head space analysis, 24 molecules proved to be well identified with a high degree of proficiency (a minimum of 50% for the identification quality index). They were associated with fruity, sweet, and vegetable flavors (Table 4) (Acree and Arn, 2012; Kim et al., 2015). A first principal component analysis (PCA), carried out with the 24 chemical compounds listed on supplement (Table 5), allowed us to find five molecules able to significantly differentiate the different samples: Ethanol, 2-butanol, p-cymene [also named: 1-methyl-4-(1-methylethyl) benzene] and 2,5-Bis[(1,1-dimethylethyl) phenol]. The fifth molecule was detected in 32 samples out of 38 but the molecules library proposed different identifications: 2-Mercapto-4-phenylthiazole or 1-aminoanthracene or 9-phenantrenamine, which was difficult to associate with chemical reactions observed in fermented products. A second PCA was performed using these five molecules: the samples (D0 + D9) were separated into two sub-groups (Figure 4). Sub-group 1 was characterised by the presence of 2-butanol for all "D9" samples, except S3 and S9. This was reflected by an increase in the relative area of the peak of 2-butanol from 0 to 2.59% on D0, to 3.48 to 17.87% on D9. S3 and S9 samples were not ground and no water was added. 2-Butanol is associated with a wine flavour and classified as a food additive – it is a flavouring agent – by the European Union. In the

literature, *Clostridium acetobutylicum* and *Clostridium beijerinckii* are frequently described as 2-butanol producing microorganisms, as well as yeasts (Lee et al., 2008; Maiti et al., 2016; Steen et al., 2008). However, the presence of Clostridia appears doubtful in our media: the redox potential is incompatible with the metabolic requirements of this bacteria. The second group of molecules encompassed the samples at D0, and S3 and S9 at D9. This group was characterised by the presence of ethanol and P-cymene. P-Cymene is described as an aromatic organic compound, used as a flavouring agent and involved in the elaboration of citrus flavors. It could originate from the carrots and probably accumulated as a consequence of the action of microorganisms (Vikram et al., 2006).

Usually, the valorisation of vegetable wastes necessitates a chemical, mechanical or physical pre-treatment to improve the production yield of interesting molecules (Laufenberg et al., 2003). And these processes can generate additional wastes, or consume large quantities of energy. These downsides have encouraged exploration into the reduction of chemical and microbial inputs (Lin and Tanaka, 2006). In our case, our results led us to deduce that the spontaneous fermentation of carrot pulps did not allow significant levels of chemical compounds to be obtained. This observation has to be examined alongside the great stability of the ecosystem highlighted previously. The experimental design probably did not go far enough in destabilising the carrot pulp to obtain interesting molecules.

Results of the experimental design

The parameters tested – culture temperature, grinding, water addition, refreshment – were analysed according to MLR procedure, the physico-chemical data being used as responses. Whatever molecule measured or step considered, the negative influence of temperature was nearly systematically observed. This means that a rise in temperature led to a decrease in the production of mainly acetic acid, and consequently titratable acidity, ethanol and to a lesser extent D-lactic acid. We can propose two

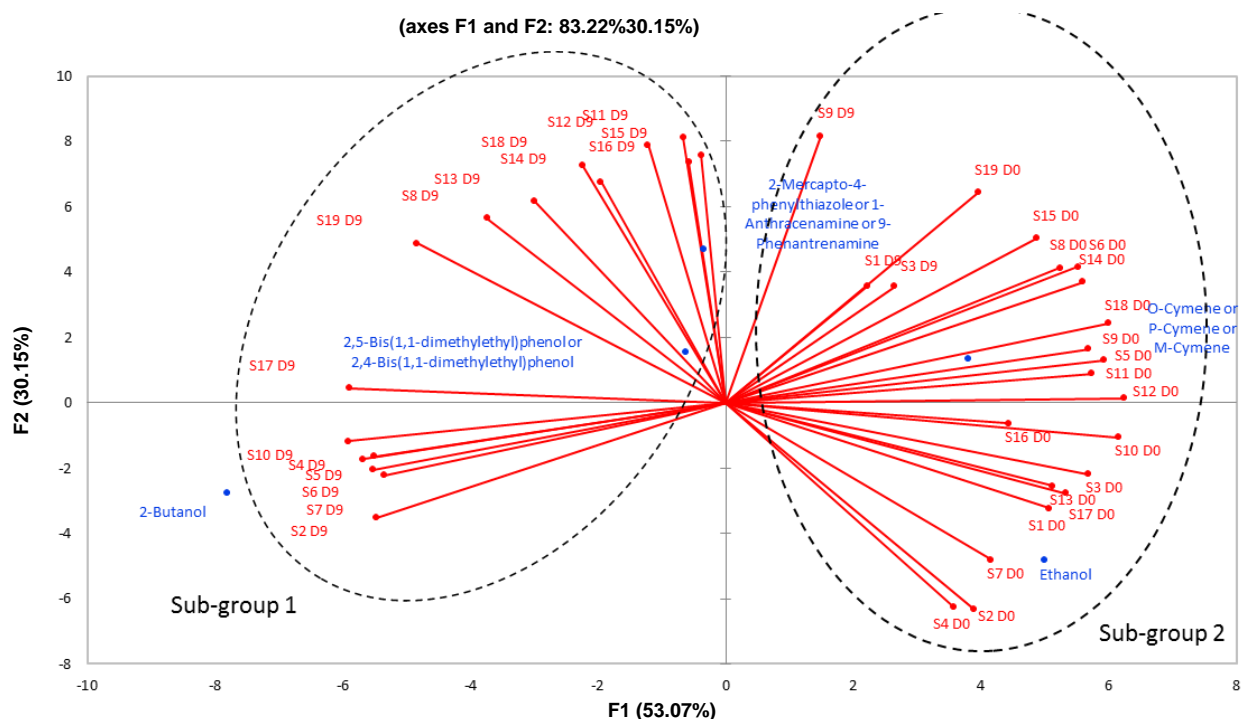


Figure 4. Principal component analysis made on the five significant molecules obtained after SPME analysis of the 19 samples analysed at D0 and D9.

Table 5. Volatile compounds identified by SPME GC/MS for different conditions of carrot pulp fermentation, at D0 and D9. Values correspond to percentages of relative area of the peak obtained. For certain compounds, several choices are proposed for identification.

| Compounds (PubChem name) | E1 | | E2 | | E3 | | E4 | | E5 | | E6 | | E7 | | E8 | | E9 | | E10 | | E11 | | E12 | | E13 | | E14 | | E15 | | E16 | | E17 | | E18 | | E19 | | | |
|--------------------------|------|------|-------|-------|------|------|------|------|------|-------|------|-------|-------|-------|------|------|------|------|------|-------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|-------|------|------|------|------|
| | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | D0 | D9 | | | | |
| Ethanol | 6.79 | 0 | 11.15 | 1.4 | 7.52 | 0 | 7.75 | 0 | 6.3 | 0 | 5.67 | 0.83 | 12.88 | 0 | 6.16 | 0.44 | 7.5 | 0 | 7.49 | 0.87 | 8.29 | 0 | 8.68 | 1.08 | 5.89 | 0 | 6.02 | 0 | 5.26 | 0 | 3.88 | 0 | 7.63 | 0.85 | 6.94 | 1 | 3.2 | 1.2 | | |
| 2-Butanol | 0.86 | 0 | 2.31 | 11.63 | 0.84 | 0 | 2.59 | 3.48 | 0.55 | 17.87 | 0.39 | 12.71 | 0 | 14.25 | 0.7 | 5.44 | 0.49 | 0 | 0.53 | 14.14 | 0.5 | 1.37 | 0 | 3.32 | 0 | 3.83 | 0.58 | 2.52 | 0 | 0.66 | 0 | 0.92 | 0 | 1.3 | 0 | 11.52 | 0 | 4.19 | 0.17 | 6.23 |
| 2,4-Dimethylquinoline | 0.98 | 0.45 | 0 | 0 | 1.15 | 0.57 | 0 | 0 | 0.87 | 1.61 | 1.03 | 2.26 | 0 | 0 | 1.38 | 2.55 | 1.67 | 0.35 | 2.49 | 3.08 | 2.25 | 1.86 | 2.25 | 2.45 | 0.74 | 1.52 | 1 | 1.8 | 1.02 | 1.48 | 0.97 | 1.98 | 1.3 | 2.58 | 0.83 | 3.04 | 1.57 | 2.9 | | |

explanations: these molecules being volatile, the amount lost to evaporation was all the greater as the temperature rose higher. But, we could also suppose that microorganisms were used to living at low temperatures (between 15 and 20°C). Consequently, their metabolism was not adapted to higher values. It is noteworthy that a strong correlation was observed between the rod shape LAB level ($r > 0.8$) and ethanol, D-lactic and L-lactic acid, and acetic acid. This means that the amount of these substances mainly resulted from the metabolic activity of LAB. In a sense, if we are looking to obtain specific compounds, we will have to reduce the influence of this microflora or promote fermentation by the controlled addition of starters. Considering the other factors, the addition of water systematically led to a significant reduction in the concentration of the products measured; we can suppose this is the result of water's diluting effect. This was particularly significant in the case of phenolic compounds: increasing the amount of water in the fermentation medium led to a concomitant diminution of these molecules. The same observation could be made for the influence of refreshing the fermentation medium. Concerning the effect of grinding, no influence was noticed.

Conclusion

In this study, we investigated the use of carrot pulps to produce molecular compounds after spontaneous fermentation. Despite varying physico-chemical conditions, the pulp ecosystem proved to be stable, with a large domination of the presumed LAB and their metabolism. As a consequence, the parameters tested (organic acids, volatile molecules...) did not change a lot or were influenced by the direct effect of the factors tested on this dominant population. 2-Butanol and P-cymene seemed frequently present. 2-Butanol was mostly found at the end of the fermentation step; and the second substance might not be associated with a known bacterial metabolism. We can argue that the conditions applied were not harsh enough to destabilise the microbial populations to promote changes of physicochemical parameters. In the future, we propose to use carrot pulps as a culture medium after addition of selected microbes known to exhibit interesting features: enzymes, aroma, biosurfactants, etc. Alternatively, we can also propose to apply harsher conditions (high temperatures, low pH) to observe the behaviour of microbial populations and its ability to produce metabolic substances.

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