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ABSTRACT

Fermented vegetables are essentially produced by spontaneous fermentation of raw vegetables, which are roughly or thinly cut, salted and incubated in an oxygen-free environment. Despite the variety of types of cutting and its potential role in the rate of diffusion of solutes from vegetable tissue, and thus the fermentation rate, the effect of this factor has been scarcely studied. Our aim was to investigate how the cutting and small variation of salt concentration impact the microbial and biochemical changes during the spontaneous fermentation of vegetables.

A 2 × 3 experimental design was set up with vegetable type (carrot / cabbage), cutting type (thin / rough), and salt concentration (0.8% / 1%) as factors. Vegetables were pressed down in 500 mL-jars and filled up with brine, and two independent jars used at 4 stages to characterise the microbial dynamics and biochemical changes by combining culturomics, 16S rRNA V5-V7 and gyrB metataxonomics, and targeted metabolomics.

Culturomics and metataxonomics results showed a similar succession of the main bacterial groups in both vegetables, with *Enterobacteriaceae* quickly replacing the initial microbiota,

further replaced within a few days by lactic acid bacteria, mainly represented by *Leuconostoc* sp. Cabbage and carrot fermentation rates quantitatively differed. Maximal *Enterobacteriaceae* counts were higher in cabbage (8 vs 7 logCFU/g), while lactic acid bacteria counts were higher in carrot (9 vs 8 logCFU/g). The acidification rate was faster in carrot (e.g. pH decreased to 3.8 in 40 h vs approx. 5.0 in 86 h in cabbage). Mannitol, lactic and acetic acids were the main metabolites produced in both vegetables, but concentrations were two-fold higher in carrot. Viable *Enterobacteriaceae* were not detected anymore after two-week fermentation, except for some roughly-cut cabbage samples. No pathogenic bacteria were detected. Taxonomic profiles varied according to the marker used, e.g. *Leuconostoc* was only detected with gyrB and vice-versa for *Clostridium*. The gyrB marker provided a markedly better resolution at the species-level (for 97% of ASV vs only 20% for the 16S marker). Significant effects of cutting type, and not of NaCl concentration, were observed. Thinly-cut vegetables globally showed a quicker fermentation compared to roughly-cut ones and exhibited a higher titratable acidity, e.g. 0.8% vs 0.3%, respectively, in grated and sliced carrot at 64 h incubation. In line, a quicker production of acids and a quicker decrease of viable enterobacteria were observed in thinly-cut vegetables, in particular for cabbage, for which the surface generated by the cutting was ~20-fold greater in shredded cabbage than in leaf cabbage. Some leaf cabbage samples displayed atypical fermentations, with the presence of particular taxa and atypical metabolite profiles with high amounts of ethanol produced. These general trends were modulated by quantitative and qualitative differences between replicate jars.

This study confirms the highly diverse microbiota of spontaneously fermented vegetables and the tight competition between *Enterobacteriaceae* and lactic acid bacteria in their colonisation, and documents for the first time the effect of the type of cutting on the fermentation rate.

Keywords *:* Fermented carrot/ sauerkraut / cutting / lactic acid bacteria / leuconostoc / enterobacteria / natural fermentation

Introduction

Fermented vegetables are traditionally consumed in central Europe and Asia, and have recently received a renewed interest in Western countries, for many possible reasons that include consumers' demand for more natural and sustainable foods and the growing proportion of vegetarian or vegan diets (Medina-Pradas et al., 2017; Thierry, Baty, et al., 2023). In Asian and Eastern countries, where fermented vegetables have been part of the tradition, a wide variety of vegetables are fermented and commonly consumed (Gänzle, 2022; Thierry, Baty, et al., 2023). Cabbage is the main vegetable used worldwide, either in mixture with other vegetables, as in the Korean kimchi or the Chinese paocai, or alone in sauerkraut in the East of France and in Germany, produced at industrial scale (Tamang et al., 2020). In Western countries, sauerkraut, olives, and cucumber are the main fermented vegetables commonly consumed. The other fermented vegetables are principally produced at domestic and artisanal scale (Thierry, Baty, et al., 2023). In a recent study we showed that French domestic and artisanal productions of fermented vegetables include a large variety of vegetables. Indeed, in the frame of a citizen science project, the 75 collected samples from citizens included 23 types of legumes, mainly cabbage (27%), followed by carrot (19%) and beets (12%), and 40% of them contained mixtures

of vegetables (Thierry, Madec, et al., 2023). The potential health effects of plant-based fermented foods have only begun to be scientifically documented, even if they make the buzz in social media without scientific support. Some vitamin concentrations can increase, or be preserved during fermentation but the effect depends on the microbial community and the conditions of production, among other factors, and contrasted results have been observed (Thierry, Baty, et al., 2023). All fermented vegetables are manufactured according to a quite simple process, which consists in cutting and tightly packing raw vegetables added with salt or brine, so that vegetables are covered with brine or with the juice released from vegetables (Buckenhueskes, 2015). Fermentation is most generally spontaneous and due to an endogenous lactic acid bacteria (LAB) community. A wide variety of recipes are used in terms of number, nature, and mixture of vegetables, and use of minor ingredients, i.e. spices and condiments (Di Cagno et al., 2013; Ashaolu & Reale, 2020).

Several bacterial groups succeed one another over time during the spontaneous fermentation of vegetables, and some of them are alive at the time of consumption. According to a meta-analysis on various fermented food covering 400 articles over 50 years, the average number of alive microorganisms in fermented vegetables such as sauerkraut, kimchi, pickles, and olives varies from 2 to 8 log colony-forming units (CFU)/g (Rezac et al., 2018). The environmental aerobic or facultatively anaerobic microorganisms first grow and are progressively replaced by a succession of heterofermentative and then homofermentative LAB. For example, in a study on carrot juice spontaneous fermentation, bacteria of the *Enterobacteriaceae* family first grew and reaching about 8 log CFU/g from the first hours of fermentation, then decreased to totally disappear after 10 days of fermentation (Wuyts et al., 2018). In parallel, during the first 3 days of fermentation, LAB actively grow, reaching around 9 log CFU/g and are responsible for rapid decrease of pH. The first LAB that grow are typically members of the *Leuconostoc* genus, followed by the *Latilactobacillus* and *Lactiplantibacillus* genera, at cell numbers of about 9 log CFU/g (Wuyts et al., 2018). Similar pictures were observed in other vegetables, e.g. in pepper (Li et al., 2024), sauerkraut (Müller et al., 2018), cucumbers (Stoll et al., 2020). Most kinetic studies on fermented vegetables are carried out over relatively short periods and do not exceed one month, which is generally considered as the final stage of fermentation since the pH is stabilised (Wuyts et al., 2018; Müller et al., 2018; Wang et al., 2020). In a recent study, carried out on 75 samples produced at a domestic scale, the age of samples ranged from 2 weeks to 4 years with a median value of 6 months. 84 % of analysed samples still contained alive LAB. LAB represented the majority of living microorganisms but also the majority of 16S reads recovered by 16S rRNA gene metataxonomics while bile-tolerant *Enterobacteriaceae* were detected in only four samples (Thierry, Madec, et al., 2023). Besides bacteria, yeasts and bacteriophages can also grow and survive in fermented vegetables (Tamang et al., 2016). Yeasts have been reported in various fermented vegetables (Liu et al., 2021; Wang, Chen, Tang, Ming, Huang, Li, Ye, Fan, Yin, et al., 2022) and were found in half of the 75 fermented vegetable homemade analysed (Thierry, Madec, et al., 2023). Given the microbial changes over fermentation time, it is crucial to combine cultural methods with culture-independent methods such as 16S metataxonomics or shotgun metagenomics, to better understand the dynamics of the microbiota of fermented vegetables. Cultural methods allow access to the living part of the cultivable microorganisms present and therefore potentially metabolically active at the time of analysis. Culture-independent methods provide access to all

the microorganisms present in the sample, whether or not they are viable at the time of analysis. The complementarity of these two approaches is all the more important as each method contains numerous potential biases (Parente et al., 2022). Concerning the 16S metataxonomics method, one of these biases lies in the specificity of the 16S primers used; the combination of several primers can make it possible to overcome these biases (Poirier, 2018; Guo et al., 2022).

Some steps are essential for a successful fermentation, notably salting and packing. The main function of salting is to withdraw water and nutrients from vegetable tissue, thus providing microorganisms with the substrates they need for growth. The NaCl concentration generally ranges from 1% to 3% of the final product. Sliced vegetables are filled and pressed in glass jars, a tight packing being crucial to eliminate air pockets and promote an anaerobic environment to limit the growth of undesirable aerobic microbiota, potentially responsible for spoilage. Products are then let to ferment at ambient temperature for at least 3 to 4 weeks before being consumed or further stored at lower temperatures. These conditions of incubation (temperature, NaCl concentration, oxygen availability, ...) determine the start-up speed of the fermentation process and thus shape the microbial community (Thierry, Baty, et al., 2023). The rate of fermentation, in particular the time course of pH decrease, is crucial to limit the growth of undesirable microbiota. Temperature and salt concentrations influence the dynamics of LAB species. The higher the temperature of fermentation is, the faster pH decreases, the earlier the dominance of (former) lactobacilli, which have a greater acid tolerance. In contrast, leuconostocs were present at a higher abundance at temperatures < 15°C (Wang et al., 2020). As for salt concentration, early studies showed its impact on the rate of acidification and the growth dynamics within the main LAB species, e.g. the growth of *Leuconostoc mesenteroides*, which is less salt-tolerant than other, homofermentative, LAB species (Pederson & Albury, 1969).

The type of cutting markedly varies in domestic productions, as recently observed in a recent study (Thierry, Madec, et al., 2023). Vegetables can be used either thinly cut (grated, shredded), or more roughly cut (into slices, dices, or simply cut in two lengthwise, for example), or even whole in the case of some small size vegetables. For example, carrot can be thinly or more roughly grated, or cut into small dices or slides, or only roughly cut. However, to our knowledge, the role of this factor has only been rarely addressed. In an original study that explored the survival of inoculated *Escherichia coli* O157:H7 and *Listeria monocytogenes* during fermentation of whole heads and shredded cabbage, both pathogens declined faster in shredded cabbage (Niksic et al., 2005). This was explained by the significantly higher total titratable acidity in shredded cabbage, compared to whole head cabbage. In summary, a thin cutting is expected to facilitate the withdrawal of water and nutrients from vegetable tissue and thus to increase the buffering capacity of juice and accelerate the rate of (lactic acid) fermentation.

Our aim was to investigate the effect of two factors, the type of cutting and a slight reduction of the amount of salt added, on the microbial and biochemical changes during spontaneous fermentation of vegetables. We chose to study a root vegetable, carrot, and a leafy vegetable, cabbage, either thinly or roughly cut. We thus compared fermentation between grated carrot and sliced carrot, and between grated cabbage and whole cabbage leaves. We also studied two slightly different salt concentrations, a concentration of 1%, which is the minimum concentration of salt normally used, and, with a view to reduce salt rates to follow health recommendations, a concentration of 0.8%. We performed fermentations of carrot and cabbage under controlled conditions and the various conditions studied were carried out in duplicate.

On these duplicates, we characterised the microbiological and biochemical changes over one month by combining culturomics, 16S rRNA gene and *gyrB* metataxonomics analysis for bacterial community, and targeted metabolomics.

Material and methods

Ingredients and experimental design

Two vegetables, carrot and white cabbage, were collectively chosen by the partners of the FLEGME project among the most frequently used in the manufacture of fermented vegetables, so as to include a root and an aerial vegetable. Non-washed organic carrots were provided by the Ferme Ty Coz farm, Saint-Pol-de-Léon, France. Non-washed organic white cabbages (*Brassica oleracea* L.), were provided by Coopérative des Producteurs Légumiers, Doué en Anjou, France. Dry (< 0,1 % humidity) grey coarse sea salt was used. Tap water was used to prepare the brine, water was boiled for carrot brine.500 g-jars with glass lid and rubber seal were used (Korken, IKEA).

A 2^3 experimental design was set up with (i) vegetable type (carrot / cabbage), (ii) cutting type (thin / rough) and (iii) salt concentration (0.8% / 1%) as factors. The two chosen vegetables, cabbage and carrot, were either thinly or roughly cut, firmly pressed down in 500 mL-jars and filled up with brine to reach final NaCl concentrations of 0.8% or 1.0%, expressed as gram of raw salt per 100 g of the mixture of vegetable and brine (**Figure 1**). More precisely, carrot was either grated or cut into slices, and cabbage were either shredded or cut into pieces of leaf of about 6-8 cm on each side.

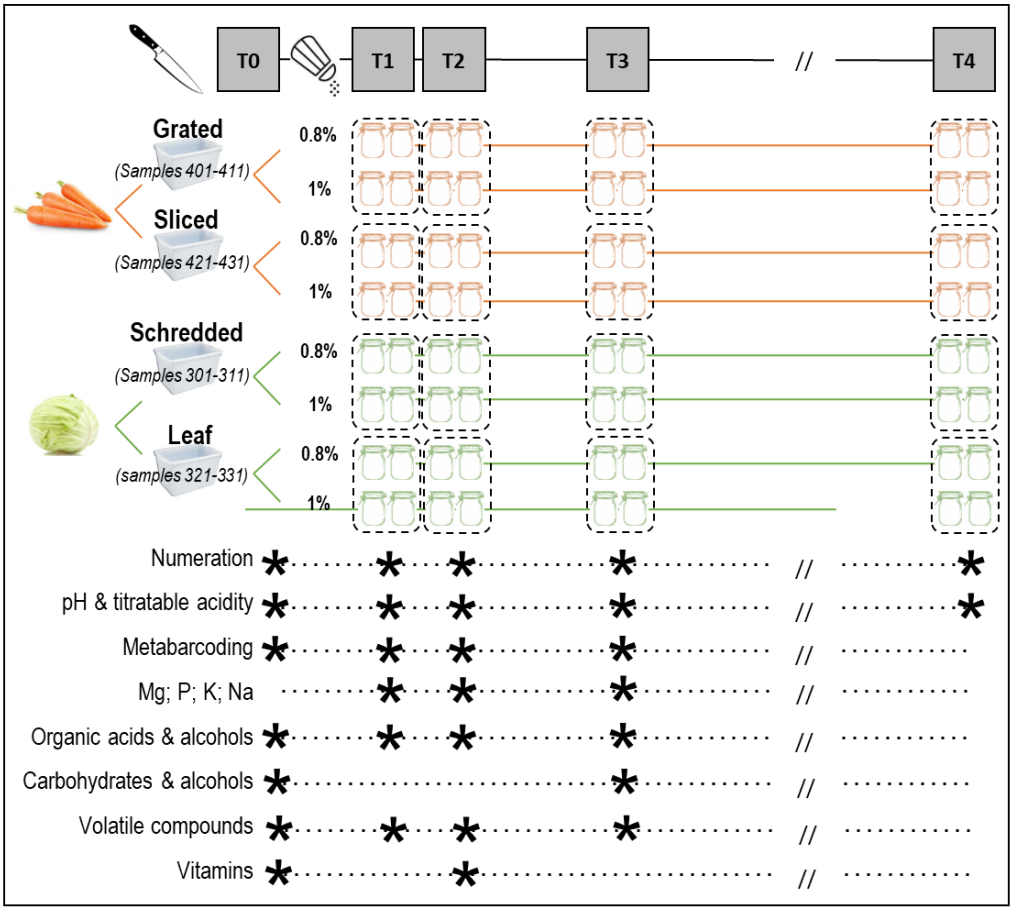


Figure 1: Experimental design used to prepare fermented vegetables and sampling. The three factors tested were vegetable type (carrot and cabbage), cutting type (either thin or rough) and salt concentration (0.8% and 1%, expressed as g raw salt per g of preparation (vegetable and brine). Sampling was done in duplicate (two independent jars) at each sampling point, represented by a star, except for volatile analysis and vitamins, done in triplicate (three independent jars), at five points: T0, initial time, T1, 1.7 day, T2, 2.7 (carrot) or 3.6 day (cabbage), T3 (4 weeks), and T4 (7 months). A few analyses were also performed at 2 weeks (stage T2b).

After removing the external leaves, cabbages were either shredded by using a professional Dito Sama TRS vegetable slicer, equipped with a 2 mm disk. Cabbage leaves were manually cut into ~9 cm x ~9 cm pieces of leaves. Then, 205 g shredded cabbage and 282 g of brine, or 232 g cabbage leaf and 246 g of brine, were successively weighed in each jar. As for carrots, they were washed, hand-peeled, grated in 3 mm pieces or cut in 5 mm-slices at the CTCPA pilot facility (agri-food technical centre and Oniris, Nantes, France). Then, 285 g grated carrot and 215 g of brine, or 285 g sliced carrot and 230 g of brine, were successively weighted in each jar. To ensure the final expected salt concentrations of 0.8% and 1%, preliminary tests were performed to determine the maximum amount of vegetables that could be packed in a jar, which varied between 205 g to 285 g according to the vegetable and the cutting types **(Supplementary Table S1),** and, consequently, the quantity of liquid (brine) that could be added (215 to 282 g), so as to calculate the salt concentration of brine in each case. The details of brine concentration are given in Supplementary Table S1.

Twenty jars of each type (vegetable, cutting type, salt concentration) were prepared, leading to a total of 160 jars. Jars were transported (1 h of transport between manufacturing site and laboratory) at ambient temperature (approx. 20°C) just after manufacturing to be incubated at the STLO laboratory at 19°C for 7 months. Two independent jars were characterised at each time point. One sample of raw vegetable was transported in a cooler (approx. 6°C upon arrival) to be analysed before fermentation (T0).

Samples were characterised for viable microorganisms, metataxonomics profiles and pH measurement at four sampling times. The first sampling was at the very beginning of fermentation, two samplings (named T1 and T2) covered the initial acidification period and the last (T3) was after four weeks incubation. Due to differences in the acidification rates, the first two sampling times were 40 h (T1) and 64 h (T2) for carrot and 40 h (T1) and 86 h (T2) for cabbage. A late sampling (T4) was performed after seven months of incubation, for microbial enumeration and pH measurement only (**Figure 1**). In addition, some intermediary sampling times were performed between T2 and T3 for volatile analysis and isolate identification (T2a, T2b, and T2c: 10, 15 and 21 days, for carrot samples and T2b and T2c: 14 and 22 days, for cabbage).

Sample names were coded as follows: 301 and 311 for shredded cabbage at 0.8% and 1.0% salt, respectively, 321 and 331 for cabbage leaf at 0.8% and 1.0% salt, respectively, 401 and 411 for grated carrot at 0.8% and 1.0% salt, respectively, and 421 and 431 for sliced carrot at 0.8% and 1.0% salt, respectively (Figure 1). For example, the sample 331-a-T3 was the replicate ‘a’ of a cabbage leaf sample prepared at 1.0% salt, sampled after 4 weeks fermentation.

Culturomics conditions

Samples of 10 g of fermented vegetables (5 g of juice plus 5 g of drained vegetables) were suspended in 90 mL of a Tryptone Salt diluent (TS, sodium chloride 8.5 g/L, tryptone 1 g/L) heated at 48°C and homogenised in a filter bag (BagPage+, Interscience), in which vegetable debris was separated from the filtrate. Microbial analyses were performed on 14 different nutritive and selective media and incubated under aerobic (air atmosphere) or anaerobic conditions (Anaerocult® A, Merck, Darmstadt, Germany) at 37°C, 30°C, or 25°C depending of the medium, as previously detailed (Thierry, Madec, et al., 2023). In brief, seven media targeted the following microbial groups: LAB, total aerotolerant bacteria, halotolerant bacteria, aerotolerant Gram-negative bacteria, yeasts and filamentous fungi, bile-tolerant *Enterobacteriaceae,* and enterococci (**Supplementary Table S2**). In addition, three media targeted spore-forming bacteria (Supplementary Table S2). *Bacillus cereus*-typical colonies on BCA were further examined, by observing their aspect on the agar medium Compass Bacillus cereus (Biokar), incubated at 30°C for 24 h and 48 h, microscopical observation and *panC* gene sequencing.

In addition, four pathogens, namely, *Escherichia coli*, coagulase-positive staphylococci (*Staphylococcus aureus*), *Salmonella*, and *Listeria monocytogenes*, were searched by a subcontracted laboratory (LABOCEA, Fougères, France), following the ISO 16649-2, ISO 6888-2, BRD 07/11-12/05, and AES 10/03-09/00 standards, respectively.

Microbial isolation and identification

To collect LAB strains, 1 to 3 isolates were picked up on the several culture media used for agar plate containing 20 to 100 colonies, according to visual aspect of the colonies (size, colour, morphology), to favour the diversity of the isolates collected. Yeast isolates were collected following the same methodology. The isolates were collected from T0 to T4 with an intermediate collection stage at 15 days which corresponds to the stabilisation of the pH. Bacteria and yeast clones were identified by 16S rRNA gene and D1/D2 domain of 26S rRNA gene sequencing, respectively. Bacterial and yeast identification were performed following the procedures previously described (Thierry, Madec, et al., 2023).

16S rRNA gene and gyrB metataxonomic analysis

DNA was extracted from the samples, using the Nucleospin Tissue kit (Macherey-Nagel, Düren, Germany) as previously described (Thierry, 2024). DNA sequences were amplified in 16S rRNA gene V5-V7 region for bacteria using primers 799F/1193R (Forward-AACMGGATTAGATACCCKG, Reverse-ACGTCATCCCCACCTTCC) and PCR conditions as previously described (Beckers et al., 2016). In parallel, the degenerate primers F64 (5'-MGNCCNGSNATGTAYATHGG-3') and R353 (5'-CNCCRTGNARDCCDCCNGA-3') were used to amplify a ~280-bp region of gyrB (Poirier et al., 2018). The 16S rRNA and gyrB amplicons were sequenced at Génome Quebec sequencing platform (Montreal, Quebec) using Illumina MiSeq PE250 technology, which generated 2 x 250 bp reads and a total of 2.45 Gb of data for amplicons.

Bioinformatic analyses

The raw sequences of 16S rRNA gene sequencing were processed as previously described (Thierry, Madec, et al., 2023). The raw sequences of *gyrB* gene sequencing were also processed using DADA2 package v 1.20.0 (Callahan et al., 2016), following the authors guidelines: we

successively applied the functions filterAndTrim, learnErrors, dada, mergePairs, makeSequenceTable, removeBimeraDenovo, and assignTaxonomy. The gyrB database from (Poirier et al., 2018) was used for the taxonomic affiliation. The amplicon sequence variants (ASV) count table, the ASV taxonomy table, and the sample metadata were combined into one phyloseq object (McMurdie & Holmes, 2013; Canon et al., 2020) for each target gene. The phyloseq (v1.44) R package was used to visualise barplots. Data were transformed into relative abundances before computing beta diversity (Bray-Curtis dissimilarity). The ComplexHeatmap (v2.16.0) R package was used to visualise the relative abundance of the different genera on a heatmap with a complete clustering based on Bray-Curtis dissimilarity computed after depth normalisation. Principal coordinate analyses (PCoAs) based on Bray-Curtis dissimilarity, calculated from the relative abundances of different genera, were conducted to evaluate the beta diversity of the samples using gyrB and 16S markers.

Biochemical analyses

The pH of juice samples was measured with a pH-metre (Hanna Instruments HI 2020-02).

Total titratable acidity (TTA), was determined on centrifuged (18000 g for 10 min at 20°C) juice samples, by titrating approximately 10 mL juice with 0.1 M NaOH to pH 8.3. It was estimated as follows: total acidity (%) = $V_{NaOH} \times 0.1 \times m / S /10$ and expressed as percentage (w/w) of lactic acid, with V_{NaOH}, volume of 0.1 M NaOH (mL); 0.1, factor corresponding to NaOH normality; m = 90, molar mass of lactic acid, S, mass of sample used (g), and was

Sample preparation

Before metabolite analysis except volatiles and vitamins, aliquots of juices were first centrifuged at 8000 g for 10 min at 4°C to eliminate vegetal debris, and the supernatant deproteinized by ultrafiltration on Vivaspin 2 centrifugal concentrator columns (polyethersulfone, 10 kDa cutoff, Sartorius) at 8000 g for 15 to 30 min at 4°C. Two chromatographic systems, High-Performance Liquid Chromatography (HPLC) coupled to UV and refractometry detection, and high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD) were combined to analyse a range of organic acids, carbohydrates, and alcohols.

Before mineral analysis, juice samples were centrifuged at 18000 g for 10 min at 4°C, and the supernatant 40-fold (for Mg and P analysis) to 1000-fold diluted (for Na and K analysis) in a 2% v/v HNO_3 (Thermo fisher scientific, Waltham, MA, USA).

For volatile metabolites, triplicate samples, taken from three independent jars, were directly analysed from juice, using headspace (HS) gas chromatography- mass spectrometry (GC-MS). Juice aliquots (2.5 mL) were placed in Perkin-Elemer 22 mL vials (B0104236, 20 mm), hermetically sealed, and the vials stored at -80°C until analysis.

Vitamin analysis was performed in drained vegetable samples stored at -20°C before analysis.

Acids and alcohol analysis by high-performance liquid chromatography

Supernatants were 2- to 4-fold diluted in a 0.005 mol-L^{-1} H_2SO_4 and stored at -20 ◦C until analysis. Lactic, acetic, citric, succinic, oxalic, and pyruvic acids, ethanol, and butanediol were quantified by High-Performance Liquid Chromatography (HPLC, Ultimate 3000, Thermo Fisher Scientific 91941 Courtaboeuf), using a Rezek ROA organic acid H + column (300*7.8 mm,

Phenomenex, California), with H_2SO_4 0.005 M as the mobile phase at a flow rate of 0.4 mL/min at 60 °C. Two detectors were used: UV detector (DIONEX-UVD 1704) operated at 210 nm and refractometer (RI 2031 Plus Jasco).

Data was processed with Chromeleon™ software. Quantification was performed using multi-standard external calibration. Standards of ethanol, butanediol, oxalic, lactic, citric, propionic, butyric, succinic, and pyruvic acids were from Merck, St. Quentin Fallavier, France, and acetic acid from PanReac, Lyon, France. Mannitol, fructose and glucose can also be analysed by this method but in the present study were not quantified because the sucrose of vegetables hydrolyses in glucose and fructose during analysis, and fructose is co-eluted with mannitol.

Carbohydrate analysis by high-performance anion-exchange chromatography

Supernatants were diluted by 400-fold in milli-Q® water (Merck, Darmstadt, Germany) and kept frozen at -20°C until analysis. Carbohydrates (sucrose, glucose, fructose, galactose, raffinose, xylose, arabinose, mannose, and mannitol) were quantified by high-performance anion-exchange chromatography (HPAEC) and pulsed amperometric detection (PAD) on an ICS-5000+ Dionex system (Thermo Electron SAS, Courtaboeuf France), as previously described (Canon et al., 2020). The system was equipped with a Dionex CarboPac PA210-Fast-4 µm column preceded by a CarboPac PA210-4 µm guard column (2*30 mm). Metabolites were eluted with KOH as eluent, at a flow rate of 0.2 ml/min with the following gradient: 0 to 32 min 13 mM, 32 min to 55 min 100 mM then return to 13 mM from 55 to 65 min. Data were acquired and processed by using the Chromeleon7™ software (Thermo Scientific). Metabolites were quantified using multi-standard external calibration (prepared at 0.1 mg/L to 40 mg/L (Merck, St. Quentin Fallavier, France).

Minerals analysis using inductively coupled plasma-optical emission spectrometer (ICP-OES)

Minerals were quantified in the initial brine and in juices during incubation using an inductively coupled plasma-optical emission spectrometer (ICP-OES) (iCAP 7200, Thermo Fisher Scientific, Courtaboeuf, France), as previously described (Martin et al., 2022). Sodium, potassium, magnesium, phosphorus, and selenium were quantified using standard external calibration. Na, Mg, K, and P standards were prepared from 100 ppm standard solutions (Reagecon, Shannon, Ireland) at 0.5 to 10 ppm in a 2% v/v HNO_3 solution, and Se at 0.01 to 1 ppm in a 2% v/v HNO_3 solution. NaCl concentrations were calculated from Na concentrations.

Volatiles analysis by headspace - gas chromatography – mass spectrometry

Volatile compounds were extracted using a Turbomatrix HS-40 trap automatic headspace sampler and analysed using a Clarus 680 gas chromatograph coupled to a Clarus 600T quadrupole mass spectrometer, operated within a mass range of m/z 29 to m/z 206 and ionisation impact of 70 eV (Perkin Elmer, Courtaboeuf, France) as previously detailed (Pogačić, 2015). Volatiles were eluted on an Elite WAX ETR column (30 m by 0.25 mm by 0.25 mm; Perkin Elmer, Waltham, MA), with helium as the mobile phase, under the following conditions: initial temperature 35°C maintained for 10 min, then increased t 5°C/min up to 230°C. Volatiles were identified by comparing their mass spectra and retention index with data from the NIST 2008 Mass Spectral Library data (Scientific Instrument Services, Ringoes, NJ, USA), from literature and from standard injection, when available. Volatiles were semi-quantified from the abundance of one specific mass fragment (m/z), in arbitrary units. Mass spectrometry (MS) data

were processed using XCMS on R software (R Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). The full width at half maximum was set to 5, the maximum number of peaks per ion to 1000, the interval of m/z value for peak picking to 0.4, the signal-to-noise ratio threshold to 6, the group bandwidth to 3, and the minimum to 0.4. The other parameters were those by default.

Vitamins

Vitamins C, K1, B9 and for carrot, beta-carotene, were quantified in vegetable samples (without juice) sampled at T0 and T3, while vitamins K2 and B12, which are not present in raw vegetables but potentially produced by bacteria, were analysed only in T3 samples. Vitamin C was analysed at Vegenov laboratory (Saint-Pol-de-Léon, France), vitamins B9 et K1 by a subcontractor laboratory, Labexia (Quimper, France), and beta-carotene and vitamins B12 and K2 were determined by another subcontractor laboratory, Agrobio (Bruz, France), using HPLC and LC-fluo internal methods.

Vitamin C was determined following NF V03-135 standard. Briefly, vitamin C was extracted from samples using a 20 g/l metaphosphoric acid solution. L(+)-dehydroascorbic acid was reduced in L(+)-ascorbic acid by using a 40 g/l L-cystein solution. L(+)-ascorbic acid was quantified by HPLC (Agilent, Les Ulis, France) with photo diode array at 265 nm as detector.

Vitamin B9 was extracted from samples, and the diluted extracts and test broth medium were put in the wells of a Vitafast B9 microplate kit, in the presence of a *Lacticaseibacillus rhamnosus* strain, and incubated in the dark at 37°C for 44 to 48 h. The intensity of metabolism of *L. rhamnosus* due to the vitamin B9 brought by the extract was measured by turbidity using a microplate reader at 620 nm and compared to a standard curve.

Vitamin K1 was determined following the NF EN 14148 standard. Fat was first eliminated by an enzymatic treatment, and vitamin K1 was measured by HPLC with fluorescent detection, after post-column reduction.

Statistical analyses

In figures 2 and 4, means and 95% confidence intervals were calculated by using the excel functions AVERAGE and CONFIDENCE.NORM, respectively.

Four-way ANOVAs were performed to determine whether the microbial and biochemical variables differed according to the vegetable, the fermentation stage, the cutting type and the NaCl concentration and the 2-way interactions, by using the R function *aov*. Means were then compared using the sidak posthoc test from the R package *emmeans*. The factors and interactions that were not significant (p-value > 0.05) were further removed from the model. When the interactions were significant, the corresponding single effects were studied on a case-by-case basis, for example the effect of the fermentation stage and the cutting type were separately tested for each vegetable. The model was also adapted for the compounds detected in only one of the two vegetables studied.

A Principal component analysis (PCA) was performed by using the PCA function of the *FactoMineR* R package to illustrate the global biochemical and microbiological composition of the fermented vegetables and the relationships between the different variables. A PCA was also done on the volatile profile for each vegetable, using the *FactoMineR* R package. Hierarchical clustering was performed on PCA data. The association of qualitative variables with sample

clusters was investigated by using v-tests and was considered significant for v-test values of <−2 or > 2.

A multi-block Partial Least Squares-Discriminant Analysis (PLS-DA) was performed to determine if samples exhibited different signatures at T3 regarding metataxonomics (16S and gyrB), metabolites (lactic acid, acetic acid, mannitol, ethanol, butanediol), volatiles and enumeration results, given their cutting type or salt concentration. The relative abundance values of a genus were retained only for the marker in which they were the highest. The function *block*.*plsda* of the R package *mixOmics* was used with two components (ncomp = 2). Outputs were visualised with the *plotIndiv* and *plotVar* R functions, and the discriminating power of the model was interpreted thanks to the *auroc* R functions, giving the AUC and Wilcoxon test p-value for each class comparison performed.

Results

Time course of microbial growth and acidification

The time-course of pH decrease and growth of the two main bacterial groups, i.e. bile-tolerant *Enterobacteriaceae* and LAB, is depicted on **Figure 2**. These three variables, as well as TTA, were significantly impacted by the vegetable studied, the fermentation stage, and, to a lesser extent, the cutting type. In contrast, no effect of the NaCl content was observed at the concentrations studied. Therefore, four replicates instead of two were available at each sampling point for statistical analyses. Marked differences of microbial counts and pH values were observed between the replicate jars sampled at each time point, but some significant trends were however highlighted concerning the time-course of fermentation.

The first bacterial group that grew was bile-tolerant *Enterobacteriaceae*, enumerated on VRBG, referred to as 'enterobacteria' below. Raw carrot and cabbage contained about 4.3 and 6.0 log enterobacteria CFU/g, respectively. Their number increased rapidly during the first days of fermentation for both vegetables. They reached 6.9 +/- 0.7 log CFU/g within 40 h in carrot samples and 8.3 log CFU/g in 40 h in cabbage samples **(Figure 2)**. Then they decreased, at a faster rate in carrot than in cabbage, and in thinly-cut vegetables than in roughly-cut ones. The enterobacteria counts were 6.6 vs 3.8 log CFU/g at 64h in sliced and grated carrot, respectively, and 8.1 vs 7.4 log CFU/g at 86h in leaf and shredded cabbage, respectively. For carrot and shredded cabbage, no enterobacteria were detected after 14 days fermentation, while for leaf cabbage, they were still detected in half samples after one-month fermentation, despite the pH decrease to 3.2 to 4.4 **(Figure 2)**. At that sampling point, 3 out of the 4 isolates from VRBG medium were identified as *Hafnia alvei*. Enterobacteria were however no more enumerable in 7-month old cabbage, in which the pH was 3.1 to 3.7 (data not shown).

LAB were enumerated on MRS, on which the 58 isolates collected were effectively LAB. LAB were present at low levels in raw carrot (3.16 +/- 0.06 log CFU/g), whereas they were below detection level in cabbage. LAB counts significantly increased over time (p-value < 0.005). Like for enterobacteria the number of LAB rapidly increased during the three first days of fermentation and reached 8.8 log in 65 h-fermented carrot regardless of the cutting type. LAB grew more slowly in cabbage than in carrot, with marked differences according to the cutting type. LAB mean counts were 7.51 and 4.56 log CFU/g in 86 h in shredded and leaf cabbage, respectively, in which they reached 8 log CFU/g only after ~5 and 13.6 days, respectively. At one

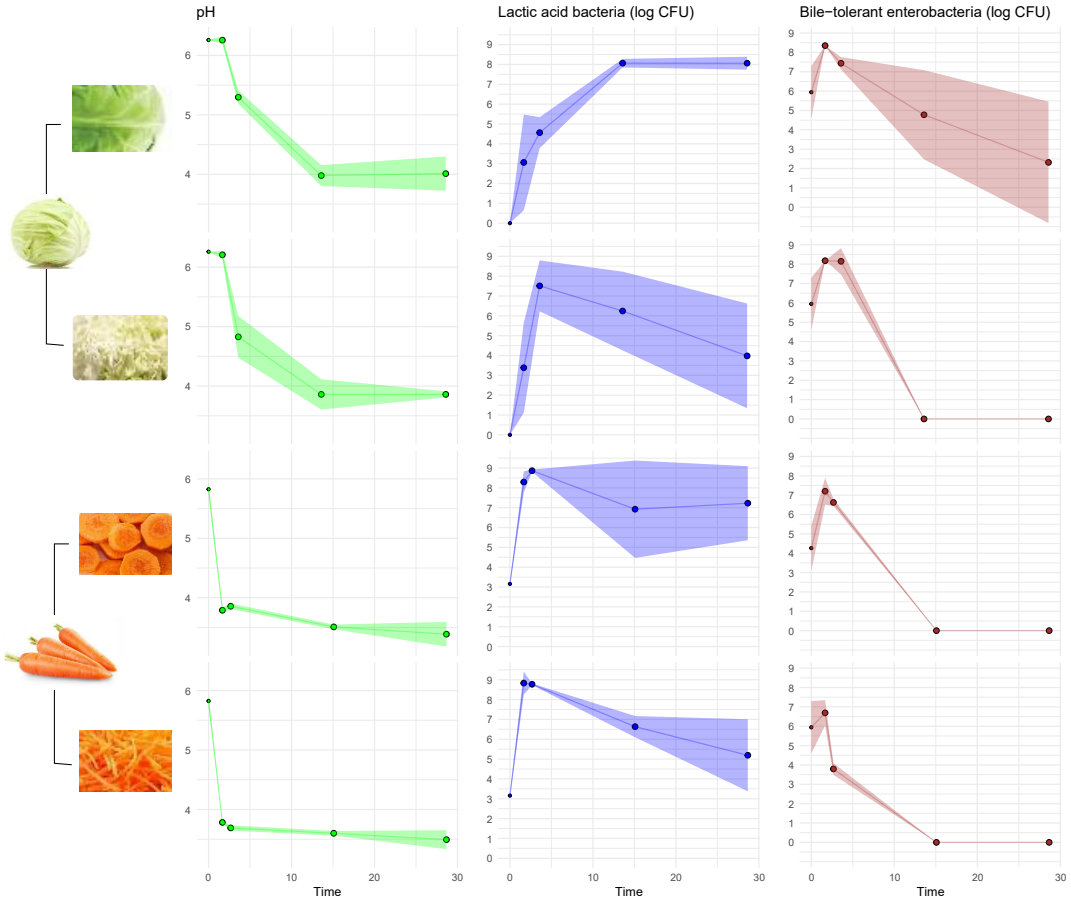


Figure 2 Time-course of fermentation in cabbage and carrot for each cutting type, illustrated by the change in pH, and the viable counts of lactic acid bacteria enumerated on MRS and bile-tolerant *Enterobacteriaceae,* enumerated on VRBG, expressed as logCFU/g. Values are means of the results observed on four independent jars, and the size of symbols proportional to the number of biological replicates (n=4, except at T0 and 15 days where n=2). The coloured areas show the 95% confidence intervals. Panels, from top to bottom: leaf cabbage, shredded cabbage, sliced carrot, grated carrot.

month (T3), LAB showed higher counts in roughly-cut vegetables than in thinly-cut ones (8.1 vs 4.0 log CFU/g in leaf and shredded cabbage, and 7.2 vs 5.2 log CFU/g in sliced and grated carrot). After 7 months LAB were still viable both in all samples with mean value of 4.3 +/- 1.1 log CFU/g. Among BL, enterococci, selectively enumerated on KF medium, were detected at low counts (< 4 log CFU/g, i.e. less than 0.04% of the total lactic acid bacteria) at the beginning of fermentation in both vegetables. They disappeared after 2 weeks fermentation, except in leaf cabbage samples, which all contained viable enterococci, ranging from 1.8 to 8.3 log CFU/g at one month. The 12 isolates collected from cabbage samples on KF were all identified as *Enterococcus faecalis,* while for carrot 7 of the 10 isolates collected were identified as *Enterococcus sp.* and 3 as other LAB genera *(Leuconostoc mesenteroides* and *Lactiplantibacillus sp.).*

The initial pH for raw material was 5.83 and 5.94 for carrot and cabbage, respectively. For carrot, the pH markedly decreased during the first two days of fermentation, with a mean value of 3.79 +/- 0.04 in 40 h regardless of the cutting type. TTA, in contrast, was highly significantly

(p< 0.0001) influenced by the stage and by the interaction stage*cutting (p< 0.01). TTA increased faster in grated than in sliced carrot, with values of 0.30% vs 0.04%, respectively, at T1 (40h) and 76% vs 0.32% at T2 (64h). TTA was 1.06 +/- 0.35 % in carrot at T3 (1 month). Afterwards, the pH only very slightly decreased, to reach 3.44 +/- 0.18 and 3.39 +/-0.30 after 1 and 7 months of fermentation, respectively, in all carrot samples. For cabbage, the pH decreased faster in shredded than in leaf cabbage during the first days of fermentation and reached 4.8 and 5.3 at T2 (86 h) in shredded and leaf cabbage, respectively. However, at this stage TTA was still very low (< 0.08%) regardless the cutting type. TTA was 0.63 +/- 0.11 % in cabbage at T3. The pH continued to decrease until about 15 days of fermentation, then stabilised to 3.94 +/- 0.21 and 3.55+/-0.13 after 3 and 7 months, respectively, in all cabbage samples.

Regarding the other media used to enumerate bacteria, the counts enumerated on BHI-YEnp, a medium used to enumerate Gram-negative aerotolerant bacteria, were very similar or a bit lower compared to the counts on VRBG, suggesting that the same bacteria grew on both these media. Accordingly, 28 out of the 30 collected isolates from BHI-YEnp medium at T1, T2 or T2b were members of the *Enterobacterales* order (*Enterobacteriaceae*, *Hafniaceae, Erwiniaceae*, *Yersiniaceae* family*)* (**Supplementary Table S3)**. Only a few carrot samples at the very beginning of fermentation showed higher counts on BHI-YEnp than on VRBG. They probably corresponded to the initial plant microbiota such as *Pseudomonadaceae* and *Erwiniaceae* members*,* the main taxa isolated from raw carrot on this medium. On the BHI-YEn medium chosen to enumerate total aerobic bacteria, *Pseudomonas sp. and Janthinobacterium sp.* isolates were identified at T0, while only LAB were isolated from T2 (Supplementary Table S3). Likewise, on the TSA-NaCl medium that targeted halophilic bacteria, 11 out of the 14 isolates collected at T3 were identified as LAB, the major part belonging to the species *Leuconostoc mesenteroides*. On the basis of the isolated and identified clones, the selectivity of KF, and VRBG media was confirmed with, respectively, 19 enterococci out of the 22 collected isolates, and 4 bile-tolerant *Enterobacteriaceae* out of the 4 collected isolates (Supplementary Table S3).

Yeasts were present on the raw vegetables, at counts ranging from 1.7 to 2.7 log CFU/g, depending on the samples. Yeasts did not develop in cabbage and disappeared fairly quickly after 4 days of fermentation. In contrast, for carrot, alive yeasts were enumerated after 7 months regardless of the cutting type. Strong variations between replicate jars were observed, with, for example at time T3 for sliced carrot, values ranging from 0 to 7.9 log CFU/g.

Regarding pathogenic and undesirable bacteria, none of the four pathogenic bacteria were detected. Some spore-forming bacteria were present at low counts in carrot samples (median value of 75 CFU/g enumerated on a rich medium, BHI-YE) and absent from cabbage except for one sample, a one-month-old cabbage sample (331-a-T3). This specific sample was also the sole that contained clostridia - 4 log CFU/g, enumerated on the selective TSN medium, and 2.44 log CFU/g on BCA medium.

A total of 191 bacterial strains (126 from carrot and 65 from cabbage) were isolated and identified to the species level by 16S rRNA gene sequencing. 97 strains were isolated during the first stages of fermentation (from T0 to T2) and 94 strains were isolated after 2 to 4 weeks of fermentation. Concerning LAB, the *Leuconostoc* genus was dominant and represented 52% of the isolates collected during the first 15 days of fermentation, followed by the *Enterococcus* (22%) and *Lactiplantibacillus* (10%) genera (Supplementary Table S3). Concerning non-lactic acid bacteria, the *Bacillus* genus dominated, followed by *Hafnia*, *Pantoea*, *Rhanella,* and

Pseudomonas genera. Concerning yeasts, 12 strains (10 from carrot and 2 from cabbage) were isolated and identified as members of the genera *Kazachstania, Rhodotorula, Saccharomyces*, *Candida, Pichia,* and *Debaryomyces*(Supplementary Table S3).

Changes in metabolite concentration

The targeted compounds, which included carbohydrates, organic acids, and alcohols, were analysed in sample juices. Concerning carbohydrates, saccharose, glucose, and fructose were the main compounds detected at the beginning of fermentation (T0). After one-month fermentation (at stage T3), the main vegetable carbohydrate detected in both vegetables was glucose, with large variation in concentrations (0 to 4.8 g/kg juice), followed by saccharose in carrot (0.5-1 g/kg juice) and fructose (0 to 0.4 and 0.1 to 1.6 g/kg juice in carrot and cabbage, respectively). Low amounts of galactose (~250 mg/L) were also detected at T3 in both vegetables and traces (< 30 mg/L) of xylose, mannose, and arabinose, and, in cabbage only, of raffinose. Concerning bacterial metabolites, five organic acids (lactic, acetic, succinic, citric and pyruvic acids) and mannitol, ethanol, and 2,3-butanediol were identified in vegetable juices, the main ones being mannitol and lactic acid, which accounted for 67 to 80% of total metabolites at T3, except in roughly-cut cabbage, which also contained high amounts of ethanol (**Figure 3)**. Two other, unidentified, compounds were detected by using HPLC-UV, at retention times 26 min and 28 min, named RT26 and RT28. In addition, oxalic acid was also detected in carrot samples. Its concentration increased from 30 mg/kg at T0 to 70 mg/kg juice at 3-month fermentation.

The concentrations of all metabolites except butanediol significantly (p-value < 0.001) increased over time, with different rates depending on the vegetable and, for some metabolites, on the cutting type. Their concentrations stabilised in juice after ~3 days in grated carrot, ~2 weeks in sliced carrot and shredded cabbage, and ~30 days in cabbage leaf, as illustrated in **Figure 4** for lactic acid, acetic acid and ethanol. Lactic acid concentrations were globally 2.5-fold higher in carrot compared to cabbage, and similar values about 2.8 g/kg were observed in 15 days-cabbage and 3-days-carrot juice samples (Figure 4). At T3 lactic acid concentrations were 2.5-fold higher in carrot compared to cabbage (5.2 versus 2.5 g/kg, respectively). Similarly, acetic acid concentration also increased over time and was higher in carrot than in cabbage (Figure 4), but it showed complex variations with significant effects of the interactions between the factors stage, vegetable (globally four-fold higher in carrot than in cabbage) and cutting type (1.4-fold higher in thinly-cut vegetables). In contrast, ethanol concentration increased with fermentation time but was mainly affected by the cutting type, with, globally 1.6-fold higher values in roughly-cut than in thinly-cut vegetables (Figure 4). For example, at T3, ethanol was two-fold more concentrated in leaf-cabbage than in shredded cabbage (3.0 vs 1.6 g/kg, respectively), and threefold more concentrated in sliced carrot than in grated carrot (2.0 vs 0.6 g/kg, respectively) (Figure 3). Mannitol was quantified only after one month of fermentation (see material and methods). At this stage, mannitol concentration was about two-fold higher in carrot compared to cabbage (9.1 versus 3.9 g/kg, respectively) (Figure 3).

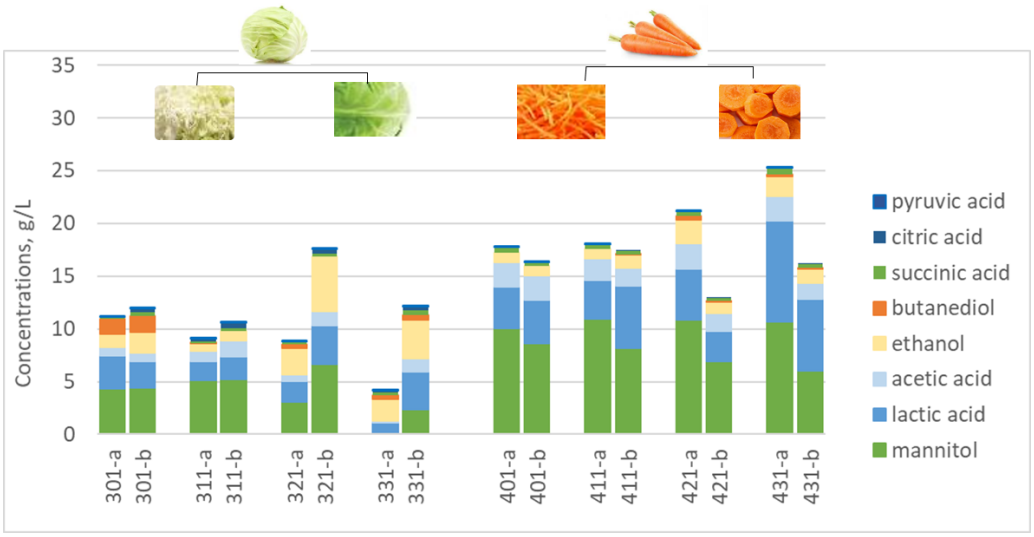


Figure 3 Concentration of the metabolites in fermented cabbage and carrot juice, expressed in g/kg, after one month of fermentation (stage T3), for each cutting type, and two salt concentrations, for two replicate jars. Sample codes: 301 and 311 for shredded cabbage at 0.8% and 1.0% salt, respectively, 321 and 331 for cabbage leaf at 0.8% and 1.0% salt, respectively, 401 and 411 for grated carrot at 0.8% and 1.0% salt, respectively, and 421 and 431 for sliced carrot at 0.8% and 1.0% salt, respectively.

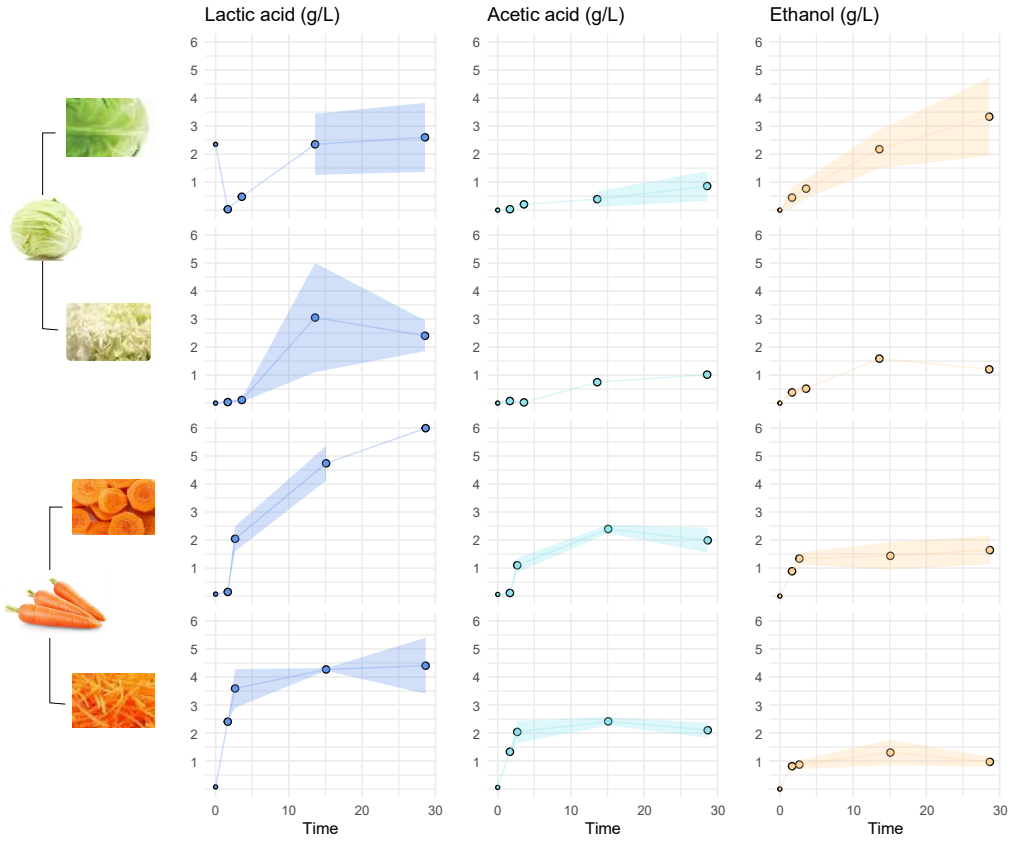
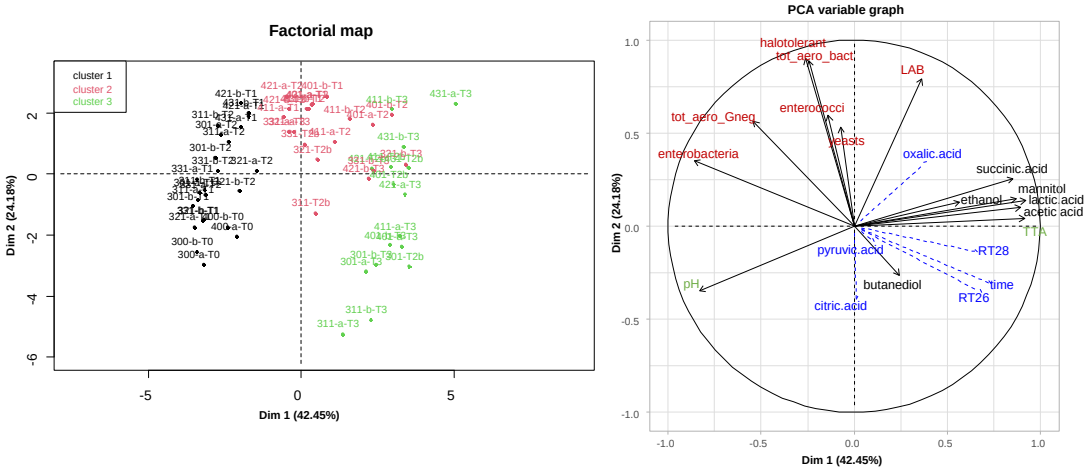


Figure 4 Time-course (day) of fermentation in cabbage and carrot for each cutting type, illustrated by the change in concentrations in lactic acid, acetic acid and ethanol, in g/kg juice. Values are means of the results observed on four independent jars, and the size of symbols proportional to the number of biological replicates. The coloured areas show the 95% confidence intervals. Panels, from top to bottom: leaf cabbage, shredded cabbage, sliced carrot, grated carrot.

Global representation of microbial and biochemical changes using PCA

A PCA was done to draw a global picture of the changes during fermentation, using 14 variables, namely the viable counts of microorganisms (n=7), the amounts of the main metabolites (n=6) and the pH values, for all cabbage and carrot samples analysed over time **(Figure 5)**. The first axis, which explains 42.4% of total variability, separates samples on the basis of pH value, negatively associated with PC1 and the concentrations in the three main metabolites, mannitol, lactic acid and acetic acid, and of succinic acid, all positively associated with PC1. PC1 was also negatively associated with enterobacteria counts. The second axis, which explains 24.2% of total variability, separates samples on the basis of high viable counts in Gram-positive bacteria, including LAB (Figure 5). Samples were clearly differentiated according to the stage of fermentation (bottom 1st panel). They were also clearly differentiated according to the vegetable and cutting type (bottom 3rd panel) but not to the salt concentration (bottom middle panel). These results show that carrot fermentation was quicker than cabbage fermentation.

Three clusters of samples were distinguished based on the hierarchical clustering performed on PCA data (Figure 5). Cluster 1, shown in black, consisted of samples at the beginning of fermentation. It was significantly (v-test > 2) associated with stages T0 and T1, a high pH, a low titratable acidity, and high enterobacteria and Gram-negative bacteria counts. Cluster 2, shown in red, characterised intermediary stage. It was significantly (v-test > 2) associated with high microbial counts on varied culture media that targeted total arerobic bacteria, halotolerant bacteria, LAB, enterococci, and yeasts. It contained many thinly-cut carrot samples. Finally, cluster 3, shown in green, consisted of samples at the end of fermentation. It was significantly (v-test > 2) associated with stages T2b and T3, a low pH, a high titratable acidity and high concentrations in metabolites, e.g. lactic and acetic acids and mannitol.



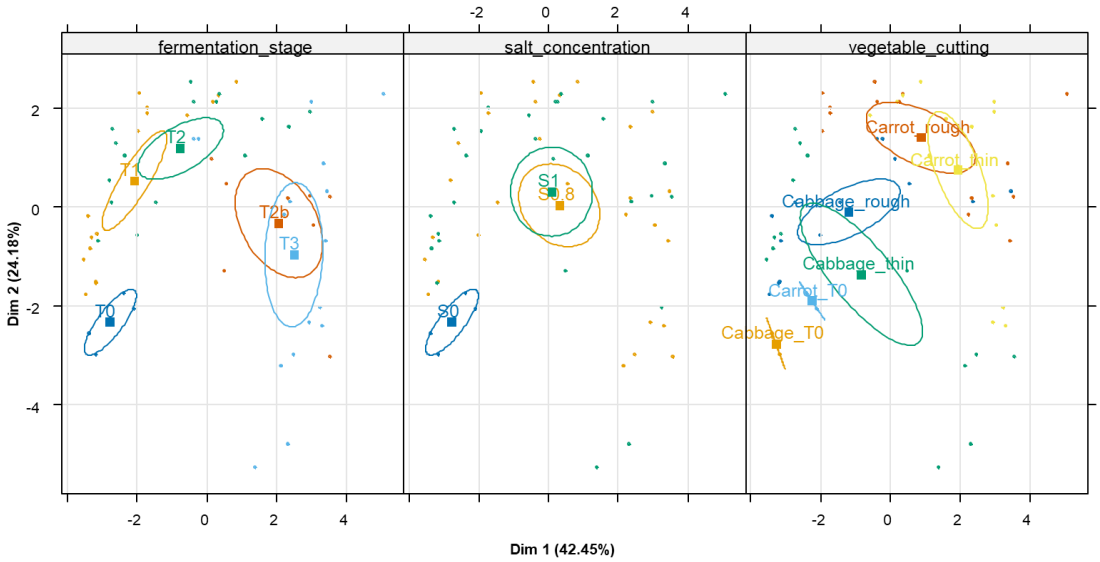


Figure 5 Principal component analysis made from the data of enumeration of viable microorganisms (n=7, shown in brown), the amounts of main metabolites (n=6, shown in black) and pH and total titratable acidity (TTA) (shown in green) for all samples (cabbage and carrot) analysed at five fermentation stages: T0, initial time, T1, 1.7 day, T2, 2.7 (carrot) or 3.6 day (cabbage); T2b (2 weeks) and T3 (4 weeks). Some minor metabolites and unknown metabolites are projected as supplementary variables (blue, dashed lines). For culture media, see Table S1; LAB, lactic acid bacteria, enumerated on MRS, enterobacteria refers to the bile-tolerant *Enterobacteriaceae* enumerated on VRBG. The individual maps (bottom panels) show the 95% confidence ellipses according to the stage of fermentation (1st panel) the salt concentration (S0.8: 0.8% or S1:1%, 2nd panel), and the type of vegetable (carrot or cabbage) and its cutting (thin or rough, 3rd panel).

Volatile compounds

In fermented cabbage, 78 volatile compounds were identified. They contained 23 sulphur-containing compounds that included 9 (iso)thiocyanates and 4 nitriles, 23 esters, 8 acids, 7 alcohols, 7 aldehydes, 6 ketones, and 4 other compounds **(Supplementary Table S4**). The abundance of 27, 15, and 10 volatiles were impacted by the stage of fermentation, the type of cutting, and the salt concentration, respectively, but in they were also interactions between these factors. The effect of the salt treatment, in particular, was always associated with interactions with the effect of stage and/or of cutting. The abundance of one third of cabbage volatiles significantly (p-value<0.01) varied during fermentation, with 27 that increased (fold change stage T3/stage T1 >2), and 9 that decreased (fold change stage T1/stage T3 >2). A PCA made to illustrate the global results on cabbage volatile profiles, showed that cabbage samples were separated on the first axis (24.1% of the total variability) according to the stage of fermentation, and on the 2nd axis (15.9% of the total variability) according to their cutting type and salt concentration (**Figure 6**). Four clusters of samples were distinguished based on the hierarchical clustering performed on PCA data (**Supplementary Figure S1**). Clusters 1, 2 and 3 were significantly associated with stages T1, T1/T2, and T3, respectively, while cluster 4 contained only one sample of roughly-cut cabbage (Supplementary Figure S1). Samples at the beginning of fermentation (cluster 1) showed high abundances of many esters, while one-month aged samples (cluster 3) contained more ethanol, butan-1-ol, acetic and butanoic acids, and

sulphur-containing compounds that originate from cabbage, such as isothiocyanates and nitriles (Supplementary Figure S1).

In fermented carrot, 52 volatiles were identified **(Supplementary Table S5).** They included 9 ketones, 9 aldehydes, 8 alcohols, 7 esters, 5 acids, 8 terpenes, 2 sulphur-containing compounds and 4 other compounds. In addition, many other terpenes and terpenoids were tentatively identified, e.g. γ-terpinene, terpinolene, 1,3,8-p-menthatriene, α-bergamotene, caryophyllene, terpinen-4-ol, β-cyclocitral, (E)-γ-bisabolene, zingiberene, cis-β-farnesene, β-curcumene, and β-sesquiphellandrene. These compounds come from carrot and were not further considered in this stud. The abundance of 32, 22, and 3 volatiles were significantly (p-value<0.01) impacted by the stage of fermentation, the type of cutting, and the salt concentration, respectively, but there were some interactions between these factors. One third of the volatiles (18 of 52) increased in concentration (p-value< 0.01; fold change T3/T0 >2). The compound with the highest fold-change was ethyl lactate (> 6000). Nine volatiles, decreased in concentration over time (p-value< 0.01; fold change T0/T3 >2), of which four aldehydes. The type of cutting also significantly (p-value< 0.01, fold-change >2) impacted the amount of 6 volatile compounds, which were all more abundant in grated carrot compared to sliced carrot. The greatest fold-changes were for two terpenes, beta-myrcene and D-limonene, suggesting that grating facilitated their release in brine compared to slicing. A PCA made to illustrate the global results on volatile profiles showed that, as observed in fermented cabbage, the samples were first separated according to their stage of fermentation on the first axis (25.8% of the total variability), and on the 2nd axis (17.1% of the total variability) according to the cutting type, and not differentiated depending on their salt concentration (Figure 6). Five clusters of samples were distinguished based on the hierarchical clustering performed on PCA data (**Supplementary Figure S2**). Cluster 1 was significantly (v-test > 2) associated with stage T1, rough cutting, and high abundance of diacetyl, cluster 2 with thin cutting and high abundance of several terpenes, such as beta-myrcene and D-limonene, cluster 3 with last stages of fermentation (T2a and T3), and many volatiles (e.g. ethyl lactate, benzaldehyde, hydroxypropanone, and dimethyl trisulfide). Clusters 4 and 5 each contained only one sample (a grated carrot at stage T2b and a sliced carrot at stage T3), characterized by pecular volatile profiles (Supplementary Figure S2).

Only 21 volatiles were shared by the two vegetables, including 7 aldehydes (pentanal, hexanal, heptanal, octanal, hepten-2-al(Z), nonanal, and benzaldehyde), 4 esters (methyl acetate, ethyl acetate, ethyl butanoate, and ethyl hexanoate), 3 alcohols (ethanol, methylbutanol, and hexan-1-ol), 3 ketones (pentan-2-one, diacetyl, and acetoin), 3 acids (acetic, octanoic, and nonanoic acids) and one sulphur-containing compound (dimethyl trisulfide).

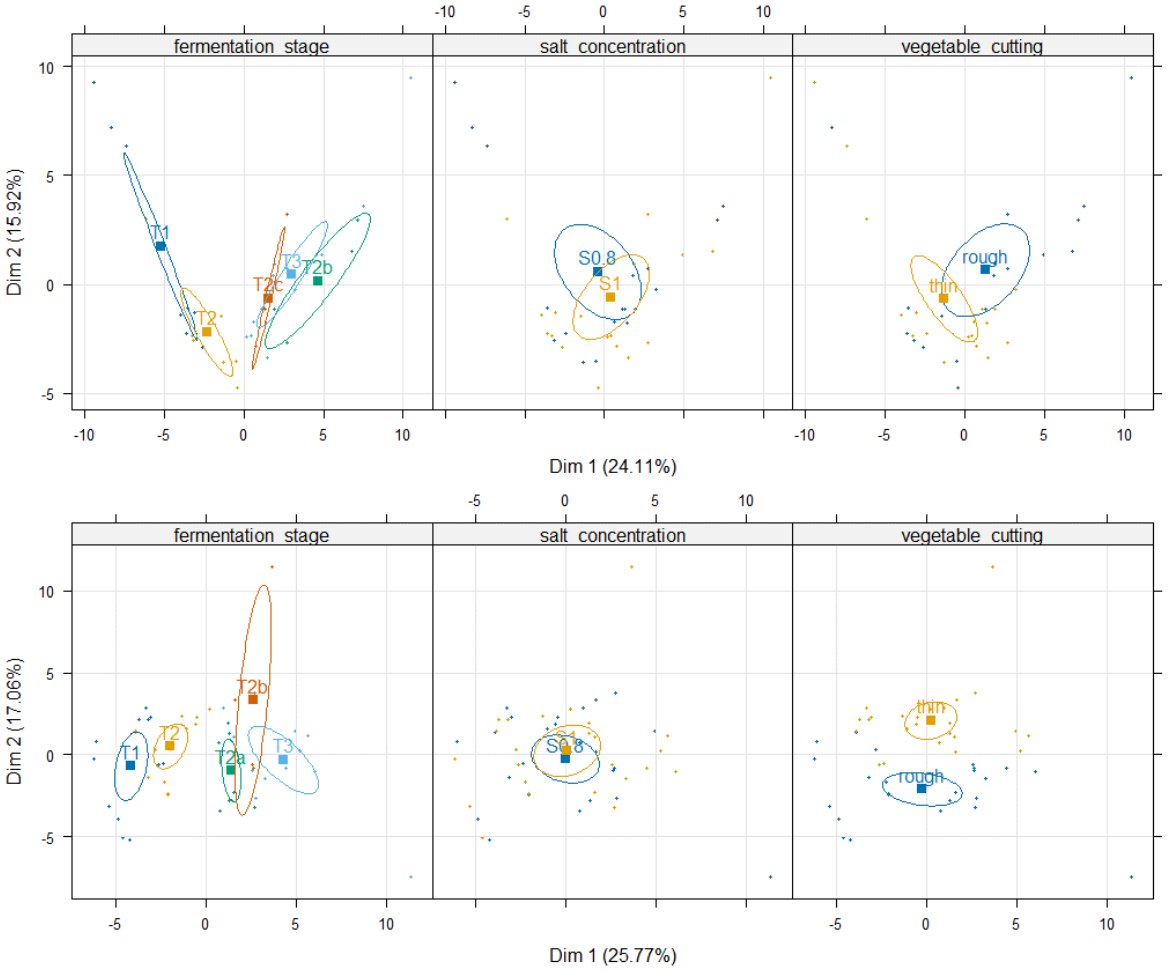


Figure 6 PCA of volatiles identified in fermented cabbage (upper row) and fermented carrot (bottom row). The three maps of individuals are coloured and show the 95% confidence ellipses according to, first panel, the fermentation stage: T1 (40 h), T2 (2.7 day for carrot or 3.6 day for cabbage), T2a (9.7 days), T2b (15 days), T2c (22 days), T3 (one month), 2nd panel, the salt concentration : S0.8: 0.8% or S1: 1%, and 3rd panel, the type of cutting: thin or rough

Mineral and vitamin concentrations

NaCl concentrations in juices, calculated from Na concentrations, significantly varied according to the amount of added salt with, on average, 9.06 g/l and 7.08 g/l in 1%-salted and 0.8%-salted samples, respectively. The differences in salt concentrations between the salt concentrations observed in juices and the targeted concentrations (0.8 and 1%) results from the composition of the coarse grey salt used to prepare the brines. Our results are consistent with the composition of coarse grey salt (e.g. 34 +/- 3 g Na per 100 g of product, i.e. 86.4 g NaCl per 100 g of product). The differences between targeted and observed salt concentrations could also result from Na migration from the brine to the vegetables during incubation, thus decreasing its concentration in the juice. Significant concentrations of Mg were also brought in the initial brine by the coarse salt: 1 g of Mg for 78 g of Na. By comparing the total concentrations in juice and the concentration brought by the salt added, we calculated that the proportion of Mg coming from salt accounted for more than half of the total Mg content in juices (59%, and

64% in thinly- and roughly-cut carrot, respectively, and 51% and 59% in thinly- and roughly-cut cabbages, respectively). We thus calculated the Mg concentration that originated from vegetables only, called 'Mg_veg'. In contrast, the coarse salt used did not bring P and K, and their proportions coming from the salt in juice were negligible (< 0.01% and < 0.09%, respectively).

The mineral content in P, K, and Mg_veg (Mg coming from the vegetable, see above) significantly (p-value < 0.001) differ in the two vegetables. Carrot juice contained 1.9 times more P, 1.7 times more K, and 20% less Mg_veg compared to cabbage juice, with average concentrations of P, K, and Mg_veg of 1793, 146, and 27 mg/L, respectively, in carrot, and 1068, 78 and 34 mg/L, respectively, in cabbage (**Supplementary Table S6**). During fermentation, the concentrations of P and Mg_veg significantly (p-value < 0.01) increased in cabbage and carrot juice, with 32% more P and 52% more Mg_veg after a 1-month incubation (T3) than at the beginning of fermentation (stages T1-T2), showing a gradual migration of minerals from the vegetable tissue to the juice. The contents in P, K, and Mg_veg were significantly (p-value < 0.001) higher in the juice of thinly-cut vegetables, with a greater effect in cabbage than in carrot. Juices from shredded cabbage contained, on average, 32%, 18% and 20% more P, K, and Mg_veg, respectively, compared to juices from leaf cabbage, while grated carrot contained 16%, 10% and 13% more P, K, and Mg_veg, respectively, than sliced carrot. The contents in P and K were also globally slightly but borderline significantly (p-value < 0.06) higher in the more salted samples, with on average, 8% and 16% more P in 1% salted carrot and cabbage juice samples, respectively, and 5% and 9% more K in 1% salted carrot and cabbage juice samples, respectively. Trace amounts of Se were also detected in carrot only, at concentrations < 0.040 mg/L.

The concentrations of the six vitamins analysed, expressed in mg or µg per 100 g of fresh drained vegetable, and the content in dry matter are given in **Table 1**.

Cabbage globally contained higher amounts of vitamins. Concentrations in vitamins C and B9 were 12-fold and 2.8-fold higher in cabbage than in carrot, on average, while vitamin K1 was detected at ~4 µg/100g in cabbage and was under the detection level (< 3 µg/100g) in carrot. Compared to raw cabbage, fermented cabbage contained about twice more vitamin C after 1 month, while in carrot, a slight but not statistically significant increase of vitamin C was observed. In contrast, vitamin B9 and beta-carotene tended (p-value = 0.010) to decrease over time. Regarding vitamins B12 and K2, which are not present in raw vegetables, they were not produced or at too low amounts to be detected in fermented vegetables. Concerning the impact of cutting size, roughly-cut vegetables were slightly, but significantly (p-value<0.03) more enriched in vitamin C than thinly-cut ones (+19% and +43% in cabbage and carrot, respectively). As for the effect of salt concentration, the content in vitamin C was slightly higher (+20%) in the less salty carrot samples, while it was not significantly impacted by salt in cabbage. The vitamin B9 decreased more in the 0.8%-salted than in the 1%-salted samples.

Table 1: concentrations of vitamins C, B9, B12, K1, K2, of beta-carotene and content in dry matter in vegetables before (T0) and after one-month fermentation (T3). Values are mean and standard deviation of duplicate analyses (except for vitamins K1, K2 and B12 for which one analysis was done)

Sample	Code	Vitamin C mg/100g		Vitamin B9 µg/100g		β-caroten mg/100g		Vit K1 µg /100g	Vit K2 mg /100g	Vit B12 µg /100g	Dry matter, %	
		mean	sd	mean	sd	mean	sd				mean	sd
cabbage T0	300-T0	10.17	0.39	16.5	2.12	nd		3.9			10.3	0.2
cabbage T3 thin salt 0.8%	301-T3	19.13	1.01	14.0	2.83	nd		<3.0	<5	<0.1	18.3	1.2
cabbage T3 thin salt 1%	311-T3	20.44	0.67	17.0	1.41	nd		3.4	<5	<0.1	17.0	1.6
cabbage T3 rough salt 0.8%	321-T3	22.25	0.53	11.5	0.71	nd		4.3	<5	<0.1	17.6	0.8
cabbage T3 rough salt 1%	331-T3	24.79	2.63	17.5	0.71	nd		6.7	<5	<0.1	17.7	1.9
carrot T0	400-T0	1.05	0.11	7.65	0.07	11.3	0.3	<3.0			9.2	0.1
carrot T3 thin salt 0.8%	401-T3	1.31	0.22	2.35	0.07	8.0	2.4	<3.0	<5	<0.1	12.6	0.5
carrot T3 thin salt 1%	411-T3	1.20	0.16	8.65	1.77	9.8	1.5	<3.0	<5	<0.1	12.9	0.1
carrot T3 rough salt 0.8%	421-T3	2.04	0.02	1.35	0.21	9.3	1.5	<3.0	<5	<0.1	13.8	0.1
carrot T3 rough salt 1%	431-T3	1.54	0.02	1.65	0.07	0.4	1.3	<3.0	<5	<0.1	12.6	0.3

vitamin concentrations are expressed in mg or µg per 100 g of fresh drained vegetable; sd: standard deviation: nd: not determined;

Metataxonomics results

The read numbers of sequenced samples ranged from 2,617 to 186,755, with a median value of 29,203 and a mean of 43,484. All samples sequenced after T0 had more than 10,000 reads. A total of 314 ASVs were obtained after 16S rRNA gene sequencing, and 2,640 ASVs after gyrB sequencing, each with an abundance exceeding 0.005% of the total. In total, ASVs belonged to 98 different genera, of which 62 detected using gyrB only, 12 using 16S only, and 24 common to both markers. The ASVs derived from gyrB sequencing enabled a higher taxonomic resolution, with 132 species identified.

The two markers were used in parallel to define the taxonomic profile of the samples at the genus level (**Figure 7**).

At T0, *Pseudomonas* largely dominated the bacterial community of both carrot and cabbage samples. Then, taxonomic profiles shifted, and *Enterobacteriaceae* became predominant (**Supplementary Figure S3**). Some genera were preferentially observed in shredded cabbage (*Aeromonas*), leaf cabbage (*Buttiauxella*), or carrot (*Erwinia*) samples, while others were present regardless of the vegetable (*Rahnella*, *Enterobacter*, *Serratia*). LAB appeared from T1 (*Leuconostoc, Lactococcus*) with *Lactiplantibacillus* or *Levilactobacillus* dominant at T3 in grated and sliced carrot (except for sample 411-a-T3). In cabbage samples, the profile greatly differed, depending on the type of cutting. *Leuconostoc* and *Latilactobacillus* were detected at T2 in shredded cabbage, while *Clostridium* developed and became the main genus in leaf cabbage (even if *Leuconostoc* was detected at T2). Therefore, the taxonomic profiles differed according to the vegetable and the cutting type, especially for cabbage, but they did not appear as impacted by the salt concentration.

The taxonomic profiles also varied according to the marker used (Figure 7, **Supplementary** Figure S3 and **Figure S4**). Some genera were similarly detected by both 16S and gyrB markers (e.g., *Lactococcus*, *Aeromonas*, *Erwinia*, *Pseudomonas*), while others exhibited differential

detection patterns according to the marker. For example, the genus *Leuconostoc* was almost undetectable with the 16S marker but which was detected at a high abundance with the gyrB marker. Conversely, the genus *Clostridium* was scarcely detectable with the gyrB marker but detected with the 16S marker.

The gyrB marker higher resolution compared to the 16S marker made it possible to refine taxonomic profiles by identifying species (Figure 7), notably those relating to *Lactobacillales* and *Enterobacterales*. Regarding *Enterobacterales*, the species *Rahnella aquatilis*, *Enterobacter sp. 638*, *Serratia sp. Leaf51* and *Pantoa agglomerans* were found in both cabbage and carrot samples. For LAB, *Leuconostoc mesenteroides*, *Lactococcus piscium*, and *Leuconostoc gelidum* were present in carrot samples from T1 onwards. At T3, *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* were mainly observed. For shredded cabbage samples, *L. mesenteroides* and *Leuconostoc carnosum* were present from T2 onwards. *L. carnosum* and *Enterococcus faecalis* were observed for leaf cabbage samples at T3. Therefore, the taxonomic profiles at the species level also differed according to vegetable and cutting type, but they did not seem to be impacted by the salt concentration. Furthermore, it is worth noting that there were pronounced differences in taxonomic profiles between the duplicate jars of the carrot and cabbage samples at T0 (coded a and b). Among the 17 *Lactobacillales* species identified by gyrB sequencing, 10 were common to the isolated bacterial strains: *L. mesenteroides, Lactiplantibacillus pentosus, Enterococcus faecium, L. brevis, L. carnosum, Latilactobacillus curvatus, E. faecalis, L. plantarum, Lactococcus lactis,* and *Enterococcus casseliflavus.* For instance, strains of *L. curvatus* were isolated from the samples 311-b-T2, 331-a-T2 and 301-a-T3 and were effectively detected with gyrB sequencing in those samples.

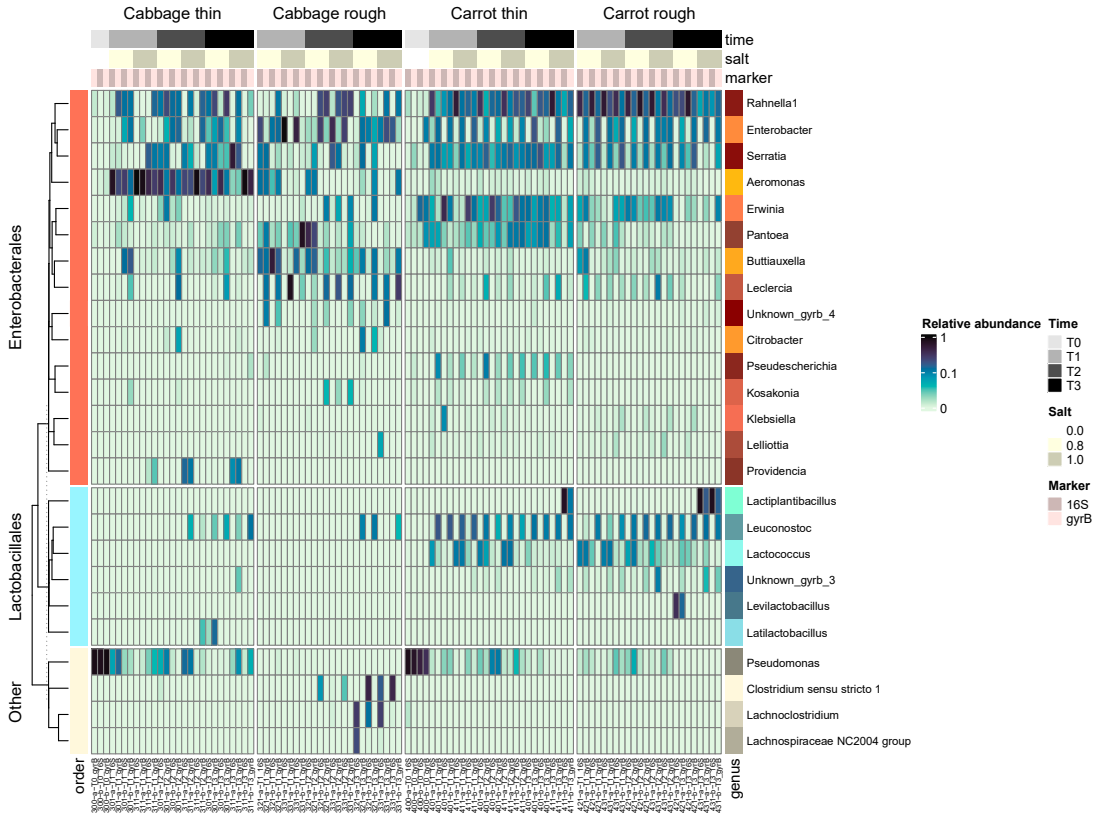


Figure 7: Heatmaps showing (A): the relative abundance of the 25 most frequently observed genera using both 16S rRNA and gyrB marker and (B) the relative abundance of the species detected with the gyrB marker. Taxa are coloured (*Lactobacillales* shown in different shades of blue, *Enterobacterales* in different shades of orange, and "other" in different shades of grey) and split according to their taxonomic order (*Lactobacillales*, *Enterobacterales* and "other") and then clustered based on the Bray-Curtis dissimilarity. Samples are organised by vegetable and cutting types. Stage and salt level are indicated by an annotation at the side of the heatmap.

A multi-block Partial Least Squares-Discriminant Analysis (PLS-DA) was performed to determine if samples exhibited different signatures after one-month fermentation (stage T3), given their cutting type or salt concentration. The datasets included gyrB and 16S metataxonomics profiles, concentration of the main metabolites from central metabolism (lactic acid, ethanol, acetic acid, mannitol, butanediol), viable counts for six bacterial groups and yeasts and some volatile compounds, selected because they result from different synthesis pathways and showed marked changes in abundance during fermentation. Models based only on cutting type did allow for the identification of a discriminant signature among the samples, unlike the models based solely on salt concentration or on both salt concentration and cutting type. The results of the PLS-DA on carrot and cabbage samples are shown in **Figure 8**. The model discriminated the samples based on the cutting type according to variables belonging to the different datasets (Figure 8).

Regarding cabbage samples, the first dimension distinguishes the samples based on cutting type across all datasets (p-value < 0.05 for all datasets, Wilcoxon test). Thinly-cut samples exhibited a relatively homogeneous signature, characterised by high concentrations of acetic acid, lactic acid and mannitol, a low pH, a higher abundance of *Leuconostoc*, and overall lower bacterial viable counts. Roughly-cut cabbage samples showed a signature marked by high ethanol concentrations, the presence of *Clostridium*, *Lachnoclostridium, Lachnospiraceae*, and a higher abundance of *Buttiauxella*. One of the replicates of rough cabbage, 331-a-T3, is a particular case with a higher abundance of *Enterococcus* and *Enterobacter*, and higher amounts of acetoin and diacetyl compared with the three other roughly cut cabbage samples and lower amounts of the main metabolites (mannitol, acetic and lactic acids).

For carrot, the first dimension distinguished samples based on cutting type only for the gyrB and metabolite datasets, while the 16S, enumeration and volatile datasets failed in discriminating the type of cutting of carrot samples at T3. Thinly-cut carrot samples had a signature characterised by higher abundance of *Pseudescherichia* while roughly-cut carrot samples having a signature characterised by a higher abundance of *Buttiauxella* and higher concentrations of lactic acid, ethanol and butanediol and a lower pH. The fermentation profiles differed between carrot samples independently of their cutting type. For example, one thinly-cut sample, 411-b-T3, showed a profile similar to that of two roughly cut samples (431-a-T3 and 431-b-T3) characterised by a lower pH, a higher abundance of *Lactiplantibacillus* and higher concentrations of lactic acid, ethanol, acetoin, and diacetyl.

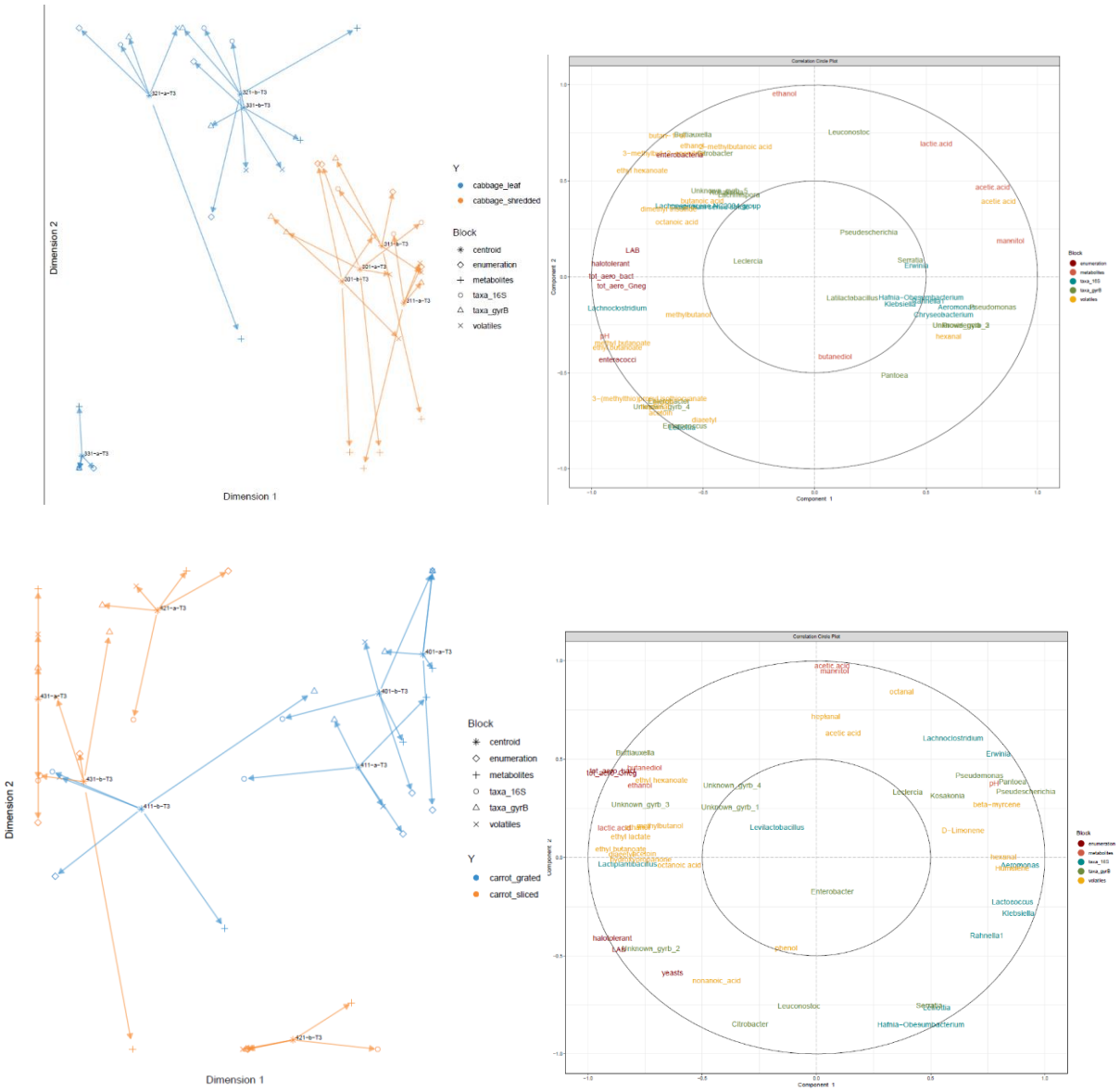


Figure 8: Multiblock PLS-DA result for cabbage (upper) and carrot (bottom) samples at T3 (one-month fermentation). The left panels represent samples from multiple coordinates to assess the alignment in the latent space. The start of the arrow indicates the centroid between all data sets for a given sample and the tips of the arrows the location of that sample in each block. The right panels represent the correlation circle between each variable and the discriminant axes as a scatter plot. The variables corresponding to the microbial viable counts for the seven following targeted microbial groups are shown in brown: lactic acid bacteria (LAB), total aerotolerant bacteria (tot_aero_bact), halotolerant bacteria, aerotolerant Gram-negative bacteria (tot_aero_Gneg), yeasts, bile-tolerant *Enterobacteriaceae* (enterobacteria), and enterococci.

Discussion

The experimental design allowed us to highlight a role of vegetable cutting despite a great variability from jar-to-jar

The objective of the present study was to better understand the microbial dynamics and biochemical changes of spontaneous fermentation of vegetables by combining several omics approaches: culturomics, 16S rRNA gene and *gyrB* metataxonomics, and targeted metabolomics. More specifically, we aimed to investigate the effect of two factors, the type of cutting and a slight reduction of the amount of salt added, on the microbial and biochemical changes during fermentation. We chose both a root and a leafy vegetable, carrot and cabbage, which are commonly used for making fermented vegetables in France (Thierry, Madec, et al., 2023). However, the experiment was not designed to address the comparison of carrot and cabbage in itself, since the vegetable cultivar, culture conditions, harvest and storage conditions (time, temperature) can also impact their fermentation (Leff & Fierer, 2013). The impact of the cutting type or size has only been rarely addressed, although this factor can markedly vary, at least in domestic productions (Thierry, Madec, et al., 2023). Regarding the content of NaCl in food, the World Health Organisation has suggested reducing sodium intake by 30% to obtain the WHO guideline of 2 g day^{-1} (i.e., 5 g of salt day^{-1}) by 2025 (World Health Organization. Regional Office for Europe, 2018). We chose to compare a salt concentration of 1%, which is the concentration of salt generally recommended, with a concentration of 0.8%, i.e. a 20% Na reduction. No significant differences in the microbial and biochemical changes were observed between these two salt concentrations, although mineral diffusion was slightly enhanced at the highest salt concentration (see below). As a direct consequence, quadruplicate samples instead of duplicate samples were available at each stage to investigate the effect of the other factors examined, i.e. the cutting and the fermentation stage. This high number of replicates was of great interest because we observed a large variability from jar-to-jar. Each jar exhibited its own fermentation path and showed some specificity regarding the microbial results, the profile of metabolites, and the acidification rate (Figures 2, 3, 4). Each jar was prepared by taking the required amount of cut vegetables from a large (~10 kg) bin, without previous mixing the bin content. Therefore, we hypothesise that the initial microbiota present in each jar differed from one another, in particular concerning LAB, which are highly sub-dominant or even under detection threshold in raw vegetables. Differences could also result from differences of composition between different carrots and different cabbages. The variations we observed were both quantitative, i.e. differences in the rate of fermentation, and qualitative, e.g. the nature of the dominant taxa that grew over time. For example, yeasts were detected in only 2 out of the 4 jars of leaf cabbage characterised at 40 h fermentation, metataxonomic profiles differed between replicates (Figure 7), and the metabolite profiles also showed quantitative and qualitative differences between replicates (Figure 3). In the industrial sauerkraut production, heterogeneity can occur in the tanks, and the juice is recirculated to avoid this source of potential defects (Pederson & Albury, 1969). Differences between replicates were also observed, but not discussed, in several studies, e.g. the results on 16S-based metataxonomics in fermented radish and carrot (Raghuvanshi et al., 2019), and in paocai (Wang et al., 2020; Wang, Chen, Tang, Ming, Huang, Li, Ye, Fan, Yin, et al., 2022). For example, the

abundance of *Enterobacteriaceae* according to the 16S rRNA gene metataxonomics ranged between ~20% and ~80% in the triplicates of four-days fermented carrot and of one-day radish, and high pH variations during the first two days of fermentation were also observed (Raghuvanshi et al., 2019). These results stress the need to include a sufficient number of replicates in studies on spontaneous vegetable fermentation, in particular for small-scale fermentation.

LAB quickly outcompeted bile-tolerant *Enterobacteriaceae* that dominate plant microbiota at the beginning of fermentation

For both studied vegetables, our culturomics as well as metataxonomic results confirm the sequential establishment of the microbial community, with the presence of a large proportion of *Pseudomonas* in raw carrot and the early development of bile-tolerant *Enterobacteriaceae, r*eferred to as enterobacteria below. The dominance of *Pseudomonas* and enterobacteria during the first hours of fermentation is consistent with the fact that these two groups constitute a large part of plant surface microbiota (Lund, 1992; Leff & Fierer, 2013; Jackson et al., 2015). *Pseudomonas* members are characterised by their great ability to colonise different ecological niches and were recovered from a wide variety of 77 samples from 11 different vegetables (Ruiz-Roldán et al., 2021). In the present study, viable enterobacteria were lower in raw carrot than in raw cabbage, 4.3 and 6.0 log CFU/g, respectively, which may be due to the fact that carrots were washed before use, in contrast to cabbages. Enterobacteria are largely represented in the microbiota of fresh vegetables, e.g. at ~5.2 log CFU/g in 41 out of the 45 samples characterised, which included carrot, cabbage, and five other fresh vegetables (Al-Kharousi et al., 2016). The first shift conserved among different fermented vegetables was from the initial microbial population of vegetables to *Enterobacterales,* according to a recent integrative bioinformatics approach used to perform a meta-analysis of 10 public amplicon data sets on fermented vegetables (Junker et al., 2024). For example, members of the *Enterobacteriaceae* family that included many non-affiliated taxa and *Erwinia* largely dominated on different vegetables, according to a 16S-based metataxonomic analysis (Raghuvanshi et al., 2019).

Our results also confirm the early development of LAB, which manage to outcompete *Pseudomonas* and enterobacteria notwithstanding their prevalence in raw vegetables. This is in full agreement with the results of a bioinformatics meta-analysis on different fermented vegetables, which showed that, after *Enterobacterales* domination, a second shift led to an assemblage dominated by *Lactobacillales*, i.e. LAB (Junker et al., 2024). This second shift was observed for example in carrot juice (Wuyts et al., 2018), cucumber (Stoll et al., 2020), kimchi (Song et al., 2020; Jung et al., 2022), and paocai (Wang et al., 2020). LAB are able to live as an endophyte in a large variety of crop plants (Pontonio et al., 2018). In our study, viable LAB were non-detectable in cabbage and their initial counts were 3 log CFU/g in carrot, in line with the values of 2 to 4 CFU/g previously reported (Di Cagno et al., 2013). Plant endophytic communities are dominated by *Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria. Firmicutes* are sub-dominant and mainly represented by *Bacillales,* while *Lactobacillales* are generally under 0.1% of relative abundance (Hacquard et al., 2015; Kõiv et al., 2019). LAB are often not detected in the starting ingredients by metatoxonomics, as shown in sauerkraut manufacture (Zabat et al., 2018). The selective pressure of the environmental conditions that result from the fermentation process applied, in particular the anaerobic conditions, indeed favours the growth

of LAB to the detriment of enterobacteria (Yu et al., 2020). LAB growth leads to a significant acidification of the environment, thus inhibiting the growth of enterobacteria, which do not withstand the acidic pH (Ostling & Lindgren, 1993). Concomitantly with acidification, we effectively observed a drastic reduction in viable enterobacteria, which were no more detected by plate counting after two weeks in 3 of the 4 conditions studied, i.e. in shredded cabbage and both thinly and roughly-cut carrot, which were characterised by a pH < 4.0 and a titratable acidity > 0.45. These results are in line with the safety threshold recommended by the Codex Alimentarius standard for pickled fruits and vegetables, which stipulates that the product has to be prepared and packed "to ensure an equilibrium pH of less than 4.6" (FAO, 2007). Our results further illustrate the ubiquitous nature of LAB in food fermentation, thanks to their ability to rapidly ferment different carbohydrates into lactic acid (Gänzle, 2015). Besides the inhibitory activity of the organic acids they produce, LAB could also inhibit enterobacteria through the synthesis of antimicrobial peptides, since many of the species found in our study are known to potentially produce bacteriocins (Zacharof & Lovitt, 2012; Hernández-González et al., 2021). A low proportion of LAB compared to that of *Enterobacteriaceae* was observed using metataxonomics in the present study, even after one month of fermentation, in particular in fermented cabbage. In a previous study using the same methods to characterise domestic samples of fermented vegetables, metataxonomics results showed that LAB had a median abundance of 90%, mainly represented by members of the *Lactobacillaceae* family, and that *Enterobacterales* was the 2nd main taxon (Thierry, Madec, et al., 2023). Moreover, a negative relationship was observed between the abundance of *Enterobacterales* and the age of the samples. The lower LAB abundance observed in the present study could thus be explained by the age of sample, one month maximum in the present study versus a median duration of 6 months (from 2 weeks to 4 years). Similarly, in a study on paocai fermented at 15°C and 25°C during one year, LAB dominated, representing about 60% of the total abundance, with (former) *Lactobacillus* as dominant genus (Wang et al., 2020).

The first LAB species that grew in both vegetables of the present study were heterofermentative species, *Leuconostoc sp.* and *L. lactis,* in agreement with previous reports in other fermented vegetables, such as paocai (Wang, Chen, Tang, Ming, Huang, Li, Ye, Fan, Chi, et al., 2022). Heterofermentative LAB are capable of degrading a wide variety of carbohydrates (Gänzle, 2015) and *Leuconostoc* efficiently metabolises sucrose (Cogan & Jordan, 1994).

The choice of the metataxonomics markers markedly impact the picture of the bacterial community. Metataxonomics using both 16S rRNA and gyrB genes as markers helped us to describe the changes in bacterial community during fermentation. The gyrB marker, although rarely used in metataxonomics, provides species-level taxonomic resolution in food ecosystems (Poirier et al., 2018), as effectively observed in the present study. For the 16S marker, the V3-V4 region is the most commonly used in the field of food microbiology (Parente et al., 2022), but we chose the V5-V7 region in the present study to compare the results with that of our previous study on fermented vegetables (Thierry, Madec, et al., 2023). Both markers confirmed the succession of *Enterobacteriaceae* and LAB, except in some roughly-cut cabbage samples. However, the gyrB marker uniquely detected *Leuconostoc* as the first LAB genus which appeared, which is also confirmed by the results of the culture-dependent approach. In three roughly-cut cabbage samples, the 16S marker uniquely detected *Clostridium* taxon. The detection of this undesirable genus is important since some *Clostridium* can be responsible for

food poisoning. We however failed in finding reports on clostridia-related poisoning associated with the consumption of fermented vegetables.

Metabolites of fermented products as markers of microbial activity

In the present study, the compounds analysed were carbohydrates, organic acids, alcohols and volatile compounds. Among metabolites, mannitol, lactic and acetic acids were the main metabolites produced, in agreement with previous reports on fermented carrot juice (Wuyts et al., 2018) and on sauerkraut (Plengvidhya et al., 2007; Tlais et al., 2022). Mannitol, lactic acid, and acetic acid respectively accounted for about 45-55%, 22-30%, and 10-12% of total metabolites in carrot and shredded cabbage after one-month fermentation. These proportions are similar to those reported in carrot juice (Wuyts et al., 2018), sauerkraut (Plengvidhya et al., 2007), and other fermented vegetables as kimchi (Jung et al., 2011). These metabolites are typical markers of LAB metabolism. Heterofermentative LAB, as the *Leuconostoc* members identified in both cabbage and carrot, convert fructose into mannitol (Wisselink et al., 2002; Martínez-Miranda et al., 2022). They also convert other carbohydrates into lactic acid, acetic acid, and ethanol, while homofermentative LAB convert carbohydrates into lactic acid as the main end-product. In our study, the ratio of lactic and acetic acids after one month of fermentation varied from one jar to another, from 1.5 to 5 in fermented cabbage and 1.7 to 4.3 in carrot. Butanediol was also detected in fermented samples, at markedly variable concentrations. Butanediol can be produced from acetoin by *L. mesenteroides* and *Latilactobacillus sakei*. For example, metatranscriptomic analyses in kimchi showed that genes encoding the pathway from pyruvate to diacetyl/acetoin and butanediol were expressed during kimchi fermentation (Chun et al., 2017; Kim et al., 2020). Concerning volatiles, most of them cannot be used as specific markers of microbial activity, because their pathways of formation are shared by many bacteria groups. For example, isothiocyanates, thiocyanates, and nitriles were detected by GC-MS in fermented cabbage samples (Table S3). These sulphur-containing compounds derive from the glucosinolates present in cabbage, which were completely degraded after one month fermentation (results non shown), as previously reported (Wieczorek & Drabińska, 2022). Even if some LAB strains can hydrolyse glucosinolates into nitriles, other bacteria like enterobacteria also do it (Mullaney et al., 2013). Moreover, the activity of plant myrosinase also results in glucosinolate hydrolysis, and is favoured by both the cutting of vegetable, which releases glucosinolates and myrosinase from separated cell parts, and by the pH decrease, favourable to myrosinase activity (Wieczorek & Drabińska, 2022). As for biogenic amines, which are mainly produced by *Enterobacteriaceae* from amino acid decarboxylation (Halász et al., 1994), they were not analysed in the present study. Total concentrations of biogenic amines can reach from ~50 to ~600 mg/kg in different fermented vegetables (Świder et al., 2020).

The fermentation rate of cabbage and carrot markedly differed in the present study

Carrot and cabbage fermentation exhibited a similar succession of the main bacterial groups, with *Enterobacteriaceae* quickly replacing the initial microbiota, further replaced within a few days by LAB, as detailed above. The main differences between the two vegetables used, in the present study, concerned the rate of fermentation. Despite a similar profile of metabolites

in carrot and cabbage, with mannitol, lactic acid, and acetic acid as main products, the concentrations produced in carrot were two-fold higher than in cabbage (Figure 3).

Many factors can be involved in the differences observed between carrot and cabbage, among which the specific microbiota of the vegetables used, the microbial changes during the processing steps. e.g. the washing of carrot vs only the removal of the external leaves of cabbage, and the composition of vegetables in macro- and micronutrients. Carrot generally contain 1.4-fold higher amounts of carbohydrates than cabbage (Anses, 2020), which likely explains why the concentrations in the main metabolites were higher in the juice of the former than in the latter one. Carrot and cabbage juice composition also differ in terms of micronutrients, which may also influence the development of micro-organisms. For example, our carrot juices contained more P and more K than cabbage, and similar amounts of Mg, while cabbage globally contained more vitamins. However, besides the type of vegetable, many other factors, such as the vegetable cultivar, its stage of harvest and storage time, and other factors, can influence their microbial and biochemical composition. As a result, the differences observed in the present study between carrot and cabbage fermentation should not be generalised.

A thin cutting favours the release of solutes and increases the fermentation rate

The cutting of vegetables before fermentation varies from thinly grated to large pieces of vegetables in domestic productions, as stated above, but the effect of cutting, to our best knowledge, has been scarcely studied. The “degree of disintegration” was cited, besides the temperature and the type of vegetable, among the factors that influence the fermentation (Buckenhuskes, 1993; Buckenhueskes, 2015). The nutrients present inside the vegetable cells must be released in the aqueous phase, i.e. juice, to be made available to the microorganisms via shredding, slicing, or only piercing, depending on vegetables (Buckenhueskes, 2015). The thinner the cutting, the higher the surface of cut plant tissue that can directly release vegetable solutes in brine, thus providing LAB with nutrients and increasing the buffering capacity of brine. Solutes can also diffuse from entire, free from injury, vegetables, as described in cucumber, but at a lower rate (Passos et al., 2005). In a study that aimed at modelling the equilibrium of solutes between the brine and entire cucumber, either peeled or not, it was shown that the diffusion coefficient of glucose was 9.2 times higher for peeled cucumber than for unpeeled one (Potts et al., 1986). The latter study suggests that the surface of cut tissue is an important factor to promote the diffusion of solutes into the brine. We therefore calculated the surface of cut vegetable, by estimating the mean dimension of the pieces of vegetables used in the present study, assimilated either to cylinders (entire, sliced and grated carrot), or to parallelepipeds (cabbage leaf pieces and shredded cabbage). Moreover, the experimental mass of vegetable and brine weighted per jar differed depending on the vegetable and the cutting, thus resulting in differences of the ratio of vegetable vs brine, which varied from 0.73 (leaf cabbage), 0.94 (shredded cabbage), 1.24 (sliced carrot) to 1.33 (grated carrot). Based on these ratios and the estimations of the surface of cut tissue, we calculated the cut surfaces per g of initial brine, which were approximately 0.4 cm^2, 8 cm^2, 9 cm^2, and 19 cm^2, for leaf cabbage, sliced carrot, shredded cabbage, and grated carrot, respectively, as detailed in **Supplementary Table S7**. In other words, the cut surface of the thinly-cut cabbage was approximately 26-fold higher than that of roughly-cut cabbage, while it was only 2-fold for the thinly-cut carrot compared to roughly-cut one. We therefore hypothesised that these marked differences of the cut surface

could, at least partly, explain why, in the present study, the two cutting types of cabbage markedly differed in the rates of acidification and viable enterobacteria decrease, in contrast to the slighter differences observed between sliced and grated carrots (Figure 2).

The hypothesis of a greater diffusion of vegetable solutes into brine for thinly-cut vegetables is further supported by our results of the mineral composition of juices. First, juices contained 18-32% more minerals (K, P, and Mg) coming from the vegetable tissue in shredded than in leaf cabbage, and 10-16% more in grated than in sliced carrot. Secondly, carrot juices contained more P and K than cabbage juice, although white cabbage is expected to be as rich in K and 1.5 richer in P compared to carrot, according to Ciqual data (Anses, 2020), thus suggesting a higher global diffusion from vegetable into juice in the case of carrot, for which the cut surface was greater. As a result, we can hypothesise that the buffering capacity of the juice was higher for thinly-cut vegetables compared to roughly-cut ones. This assumption is consistent with the fact that, for a given pH, TTA was higher in thinly-cut vegetable. For example, TTA was twice as high in grated carrots at T1, compared to sliced carrots, despite a similar pH. The effect of cutting was previously studied to compare the survival of pathogenic strains during fermentation of cabbage either kept as whole heads or shredded (Niksic et al., 2005). The authors attributed the lower survival observed in shredded cabbage to the significantly higher total titratable acidity in shredded cabbage juice compared to the one of whole head cabbage, in relation to the higher buffering capacity of the juice. The importance of the buffer capacity of vegetable juice has been previously highlighted. A pioneer study showed that the composition of cucumbers depended on their size, the. Smaller fruit, however, contain lower levels of sugars and a higher natural buffering capacity than larger ones and achieved a complete sugar utilisation during fermentation (Lu et al., 2002). Buffer models were later developed in cucumber juice of different composition, so as to be capable of assessing the relationship between pH and the concentration of acids (Breidt & Skinner, 2022).

The addition of salt is known to withdraw nutrients from the vegetable tissue towards the juice. We effectively observed, in the present study, a slight but significant effect of the amount of added NaCl on the release of P and K in the juice (+ 5-8% in carrot, and + 9-16% in cabbage).

In the case of leaf cabbage, which had by far the smallest cut surface, we also observed faulty fermentation. For example, on the four jars of leaf cabbage characterised after one month fermentation, two still contained alive enterobacteria (321-a and 331-b), the third (321-b) contained lower viable LAB counts (7.6 log CFU/mL) and a high content in ethanol (5.2 g/mL vs 1.6 g/mL in all other samples at that stage) and for the fourth one (331-a), the whole lactic population consisted of enterococci, associated with an atypical metabolite profile without mannitol (Figures 2, 3, and 4) and a distinct volatile profile, with for example the highest amounts of esters of butanoic acid. These four samples also contained *Clostridium* (321b, 331a, 331b) and/or *Lachnoclostridium* (321a, 321b, 331a) taxa. The mean pH of these four samples was 4.0, versus 3.6 at the same stage in all other samples. It is noteworthy to mention that many leaf cabbage samples had a very unpleasant and atypical smell. We therefore hypothesise that the cutting of cabbage into large leaf pieces was insufficient to provide LAB enough nutrients and that there could exist a threshold cut surface under which a rapid lactic fermentation is hardly achieved.

In brief, a fine cutting, besides salting, favours the release of solutes towards the juice, thus providing microorganisms the nutrients they need to grow, and increasing the buffer capacity

of brine. Consequently, a quicker acidification, a higher titratable acidity, and quicker decrease of the number of viable enterobacteria can be expected, as effectively observed in the present study in particular for cabbage, for which the surface generated by the cutting step was much greater (> 20-fold) in shredded cabbage than in leaf cabbage. The difference of salt concentration also intensified the release of solutes, as observed in the present study for some minerals, but the possible consequences on microbial growth were probably masked by the variability observed from jar-to-jar. In line with the importance of the buffer capacity, the targeted values of both the pH (< 4) and titratable acidity (1%) are given in the specifications for the protected geographical indication of "Sauerkraut of Alsace", to satisfy safety and sensory property requirements (EU Commission implementing regulation, 2018).

Some health benefits and risks associated with fermented vegetables consumption

Besides the preservation of vitamins, discussed below, fermented foods have been associated with potential health benefits, which result from two main factors. The first is the microbial production of metabolites of interest for human nutrition, e.g. vitamins, bioactive peptides, or that can positively affect human health, e.g. mannitol, γ-aminobutyric acid (Lenhart & Chey, 2017). The second is the presence of live microorganisms that can interact with the intestinal microbiota (Rezac et al., 2018). Initially much explored for dairy-based fermented foods (Kok & Hutkins, 2018; Companys et al., 2020) and the Korean kimchi (Cha et al., 2023), this is now also well demonstrated for other non-dairy foods (Wuyts et al., 2020; Valero-Cases et al., 2020).

On the other hand, in the case of poorly controlled production, the consumption of altered fermented products can lead to health risks. Concerning fermented vegetables, the high number of enterobacteria present at the beginning of fermentation is associated with the risk of toxin and biogenic amine production. The presence of spore-forming bacteria, among which toxin producers such as some *Clostridium* sp., has also been reported. In a recent South Korean quantitative risk assessment study for *Clostridium perfringens* foodborne illness via kimchi consumption, the authors conclude that the risk was "very low" (Choi et al., 2020). Enterobacteria disappear as soon as the pH is low enough for a period of time, and not all of them present hazards. For example, in the present study, we mainly identified *Hafnia alvei* among the clones isolated on the VRBG medium from the leaf cabbage samples in which enterobacteria were alive after one-month fermentation. *H. alvei* is commonly isolated from and even used as an adjunct culture in raw milk Camembert cheese. One of the leaf cabbage samples also contained clostridia. Fortunately, all these samples would not have been consumed because they had a very unpleasant odour.

We focused in this study on the vitamin content in raw vegetables and after fermentation. According to literature, the final concentration in fermented vegetables of the vitamins first depends on their initial concentration in raw vegetables, even if fermentation can result in variations in their vitamin content. The vitamin concentration has been shown to decrease during fermentation in most of the reported cases, as stated in a recent literature review (Thierry, Baty, et al., 2023). The contents in vitamins C and K1 and beta-carotene in raw carrot and/or cabbage observed in the present study are in accordance with the expected values from nutritional food tables, while the vitamin B9 content was in the lower range of the reported values (Anses, 2020). We only observed an increase for the vitamin C content during

fermentation, which may be due to microbial activity of ascorbigen degradation (Berger et al., 2020). In contrast, vitamin B9 content was not improved, in agreement with previous report (Jägerstad et al., 2004). Vitamins K2 and B12 are absent from raw vegetables and can result from microbial synthesis. Vitamin K2 is mainly present in fermented foods (Walther & Chollet, 2017) and can be synthesised by some LAB strains (Capozzi et al., 2012). Vitamin B12 is present in foods of animal origin (National Institutes of Health) and can also be synthesised by some bacteria as propionibacteria (Falentin et al, 2010). We did not observe the production of vitamins K2 and B12 in the present study. In the context of human nutrition, vitamin contents were expressed in the present study per quantity of fresh matter of fermented vegetables (without brine), i.e. as they are generally consumed. In our study, cabbage and carrot remained a good source of vitamin C and beta-carotene, respectively, after fermentation, since they bring 24 to 31% of nutritional value of reference in vitamin C for 100 g of fermented cabbage and 167 to 204 % of pro-vitamin A (in beta-carotene form) for 100 g fermented carrot.

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Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data, scripts, code, and supplementary information availability

The sequence data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI and are available online: PRJEB79032 on the webpage hosting the data https://www.ebi.ac.uk/ena/browser/view/PRJEB79032.

Supplementary information including data, and scripts is available online: DOI of the webpage hosting the supplementary information https://doi.org/10.57745/MJWSJQ; Thierry et al., 2024.

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