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► To cite this version:

Laura Lavefve, Natacha Cureau, Lindsey Rodhouse, Daya Marasini, Laura Walker, et al.. Microbiota profiles and dynamics in fermented plant-based products and preliminary assessment of their in vitro gut microbiota modulation. *Food Frontiers*, 2021, 2 (3), pp.268-281. 10.1002/fft2.75 . hal-03214622

HAL Id: hal-03214622

<https://hal.science/hal-03214622v1>

Submitted on 8 Jul 2021

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RESEARCH ARTICLE

Microbiota profiles and dynamics in fermented plant-based products and preliminary assessment of their in vitro gut microbiota modulation

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Abstract

Consumption of fermented food has long been associated with health benefits, but there is still limited knowledge on the bacterial dynamics in plant-based food fermentation outside of culture-based studies. Different fermented plant-based products were assessed for the presence of Archaea and their microbiota bacterial dynamics during the fermentation. Archaea were consistently detected in the brine of the vegetables, and constant increase in gene copy number throughout the fermentation of kraut indicated that Archaea were not only viable but actively growing. The plant-associated bacterial microbiota of cabbage and jalapeno were dominated by Proteobacteria, specifically *Pseudomonas* (51% and 39% respectively), while the okra harbored roughly equal numbers of firmicutes and proteobacteria. In cabbage and jalapeno fermentations, lactic acid bacteria (LAB), which were detected in extremely low levels in raw products, became dominant with expected succession of heterofermentative and homofermentative species. These two stages were not detected in the fermentation of okra, and *Lactobacillus* remained the most abundant genera. The kombucha fermentation was dominated by *Gluconacetobacter* as reported previously, but also characterized by high abundance of *Bacteroides*. Intriguingly, the microbiota composition and dynamics were very different between the two kombucha batches tested, suggesting redundancy in microorganisms' fermentative roles. Finally, a preliminary in vitro fermentation study was indicative of a potential bifidogenic effect of microbial metabolites from kombucha. Collectively, these data indicate that fermented plant products harbor a highly diverse microbiota, bacteria, and archaea, even after the end of the fermentation.

KEYWORDS

archaea, fermentation, fermented vegetables, *in vitro* fermentation, jalapenos, kombucha, microbiota, okra, sauerkraut

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1 | INTRODUCTION

Fermentation is one of the oldest food preservation methods. There is evidence that a rice-based fermented beverage was consumed in 6000 BC in China (McGovern et al., 2004). Cucumbers were fermented around 2000 BC in the Middle East, and the first cabbage to undergo fermentation was a kimchi (from cabbage *Brassica rapa*) in Korea. Fermented foods lost popularity during most of the last century due to the advent of modern food safety and preservation approaches (Marco et al., 2017). However, fermented foods, in particular nondairy fermented products are gaining renewed interest, therefore becoming an increasingly important commodity (Freire et al., 2017; Marco et al., 2017). Nowadays, the most popular fermented vegetables in Western countries are cucumber pickles, olives, and sauerkraut (from *Brassica oleracea*) (Breidt et al., 2013; Lavefve et al., 2019). Nonalcoholic fermented beverages are also gaining popularity. For example, kombucha is the result of the fermentation of brewed tea by a SCOBY (symbiotic colony of bacteria and yeast), resulting in a sour and carbonated beverage. The abundant health benefits associated with fermented foods and beverages are commonly attributed to a pre- or probiotic effect, though not all those claims are supported by scientific data. The lack of literature and the severe international regulation only allows a restricted number of products to be officially labeled as pre- or probiotics (Marco et al., 2017; Mokoena et al., 2016).

Modern food processing knowledge and technologies allow fermented foods to be produced with very low spoilage and risks of food-borne pathogen contaminations (Marco et al., 2017). However, by their very nature, fermented foods are characterized by the presence of diverse and relatively abundant microbiota, which are generally recognized as safe (Zhang et al., 2016). In most cases, there is little knowledge on their precise composition, as they have been traditionally tested by culture-dependent methods (Freire et al., 2017; Greppi et al., 2017) or molecular fingerprinting methods (Garofalo et al., 2017). In addition to some unsupported probiotic claims, fermented foods have been proposed as potentially prebiotic, based on the assumption that microbes perform a “predigestion” of complex dietary elements (Hill et al., 2017). Nonetheless, fermentative abilities are highly dependent on each microbial species’ specific metabolic properties, thus leading to highly variable metabolic potential based on microbial communities’ composition (Bolca et al., 2013; Wu et al., 2012).

The microbiota most commonly associated with fermented foods includes bacteria and yeasts, while Archaea are rarely considered as fermenting agents. There have been, however, several reports of halophilic Archaea found in fermented and salted food products (reviewed in Lee, 2013). It has generally been hypothesized that these Archaea are seeded from the salt itself, rather than being present in the raw food product (Henriet et al., 2014). Many novel species of Archaea have been isolated from fermented fish (Yachai et al., 2008), seafood (Roh & Bae, 2009; Roh et al., 2009; Roh et al., 2007; Roh et al., 2007; Yim et al., 2014), and canned herring (Kobayashi et al., 2000). However, there has not yet been any report of Archaea detected in foods other than salt-borne halophiles.

Here, archaeal and bacterial microbiota composition were characterized in fermented foods produced under small scale processing practices. Distinct microbiota and dynamics are described in three fermented vegetables products, mainly composed of cabbage, jalapenos, and okra, before and during the fermentation, as well as in kombucha, a fermented tea. Finally, a preliminary vitro gut microbiota experiment was conducted to assess the potential prebiotic or probiotic properties of the kraut product and the kombucha.

2 | MATERIALS AND METHODS

2.1 | Fermented food origin and collection

Fermented vegetables were provided by local small producers at the Arkansas Food Innovation Center (AFIC) housed in the Food Science Department at the University of Arkansas, Fayetteville. Three different products were analyzed:

1. A crunchy kraut containing 28% of red cabbage, 28% green cabbage (*Brassica oleracea*), 28% carrots (*Daucus carota* subsp. *sativus*) and 16% Jicama (*Pachyrhizus erosus*).
2. Pickled jalapenos containing 80% of sliced jalapeno peppers (*Cap-sicum annum*), with low amounts of carrots (15%) and onions (5%) added.
3. Whole okra (*Abelmoschus esculentus*).

The vegetables were fermented at room temperature, in the brine solution (between 2% and 4% salinity; food-grade salt from mines sources). The process was conducted for 17 days for the kraut, 15 days for the jalapenos, and 21 days for the okra. The end of the process was determined by a significant pH drop as measured by the producers. Duplicate samples of the brine (from two different fermentation vats) were taken at day 0 (T0, microbiota from unfermented vegetables), every 7 days of the fermentation (T7 and T14), and at the end of the fermentation just before packaging (TF). After this step, the sealed jars were stored at 4°C and a final sample was collected after 5 days (TF+5). All samples consisted of 2 mL of brine that were collected in sterile tubes and stored at –20°C before further analysis.

Kombucha was produced in a continuous fermentation design; with each batch fermentation lasting one week. Every 7 days, half of the fermented tea was removed to be bottled and replaced by a new batch of nonfermented black tea with addition of white cane sugar. The fermentation was conducted at room temperature. Samples of the tea were collected during two consecutive weeks (W1 and W2) from the non-fermented tea (NF), at day 0 just after adding the tea to the previous batch (T0, bacterial microbiota from unfermented tea), day 1 (T1), day 3 (T3, only for the first week), and day 7 (T7) from two fermentation vats. 2 ml of tea were collected each time in sterile two ml tubes and stored at –20°C.

2.2 | In-vitro fermentation study

The impact of kombucha and the kraut mix on the gut microbiota was studied through in vitro fermentation experiments. A sample of the final kombucha product (from the second batch), after the regular one-week length of fermentation was collected. A first tube of kombucha was directly stored at -20°C until further use as a treatment in the batch fermentation. Another tube containing 25 ml of kombucha was centrifuged (10 min at 4000 rpm, 4°C to separate the pellet and supernatant. The pellet was resuspended in 14 ml sterile water while the supernatant was transferred to a different tube. The kraut brine was collected from the jar previously kept at 4°C for 5 days after the end of the fermentation (TF+5). One part was directly stored at 4°C while another 50 ml were centrifuged (10 min at 4000 rpm, 4°C). The supernatant was discarded, and the pellet resuspended in 28 ml sterile water.

Stool samples were collected from one volunteer (Female, Caucasian, 23 years old BMI 21.4, Fasting blood glucose 94.5 mg/dl) enrolled in a separate study (Ashley et al., 2019) under a protocol approved by the University of Arkansas Institutional Review Board (IRB# 17-02-433). In vitro fermentations were setup as described before (Ashley et al., 2019; Pham et al., 2017). One liter of anaerobic fermentation medium was composed of yeast extract (2 g; Alfa Aesar, Ward Hill, MA), peptone (2 g; Fisher Scientific, Waltham, WA), bile salts (0.5 g; Oxoid, Hampshire, UK), NaHCO_3 (2 g), NaCl (0.1 g), K_2HPO_4 (0.08 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g), $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01 g), L-cysteine hydrochloride (0.5 g; Sigma, St. Louis, MO), vitamin K (10 μl ; Sigma), bovine hemin (50 mg; Sigma, Tween 80 (2 ml), and 0.025% (w/v) resazurin solution. To prepare fecal slurry, 2 g fecal sample was added to 20 ml phosphate-buffered saline, vortexed to homogenization, and filtered through four layers of cotton gauze. Within an anaerobic chamber, 14 ml of sterile fermentation medium were inoculated with 1 ml of fecal slurry and treated with either no substrate or one of the five products (kombucha, kombucha supernatant, kombucha pellet, sauerkraut brine, and sauerkraut brine pellet). After 4 h of stabilization at 37°C , 1 ml of the fermented food products were added to the tubes and a first sample was collected (T0) in a 2 ml tube previously flushed with nitrogen. The cultures were kept at 37°C and the samples stored at -20°C after addition of 0.2 ml of 2 M KOH stop solution. Three more samples were collected after 4 (T4), 12 (T12), and 24 (T24) h of fermentation and stored at -20°C after addition of 0.2 ml of 2 M KOH stop solution before performing DNA extraction.

2.3 | Microbial DNA extraction

All the DNA extractions were performed using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) with few modifications. Prior to the extraction, the 2 ml samples were centrifuged at 13,300 rpm for 3 min. The pellet was washed three times with sterile water to remove extracellular DNA. The pellet was then resuspended in 1 ml of InhibitEX Buffer and incubated at 70°C for 5 min (Analog Heat-block, VWR, Radnor, PA) to lyse the bacterial cells and separate the

DNA from DNA-degrading substances. The mixture was then added to a tube containing zirconia and silica beads (0.1 g of 0.1 mm diameter and 0.1 g of 0.5 mm diameter) and bead-beated using a FastPrep-24TM (MP Biomedicals, Santa Ana, CA) for one minute. The DNA was then extracted following manufacturer's recommendations. The DNA was eluted from the column into a 1.5 ml tube in 50 μl of elution buffer instead of 200 μl . The concentration of the purified DNA was assessed using Nanodrop (Thermo Scientific) and DNA extracts were stored at -20°C .

2.4 | Bacterial PCR

Universal PCR was used to confirm the presence of bacteria in the extracted samples using universal 16S rDNA gene bacterial primers 8F and 1541R (ALJahdali et al., 2017). The PCR reaction was composed of 12.5 μl of GoTaq (Promega, Madison, WI), 7.5 μl of sterile water, 1 μl of forward primer and 1 μl of reverse primer. Three microliters of DNA were added to the master mix to obtain 25 μl PCR reactions. The PCR program was as follows: 3 min of initial denaturation at 95°C followed by 35 cycles consisting of denaturation at 98°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1 min; and 5 min of final elongation, using a MasterCycler (Eppendorf, Hamburg, Germany) thermocycler.

PCR products were analyzed by gel electrophoresis. A 2% agarose gel was made by mixing agarose (Fisher BioReagents, Pittsburgh, PA) in TAE 1X buffer (50X from Amresco, Solon, OH) and adding 1 μl of SYBR Safe DNA stain (EDVOTEK, Washington, DC). Five microliters of PCR product were loaded into each well of the gel and separated for 45 minutes at 120 Volts, alongside a 200 bp DNA ladder (Omega Bio-Tek, Norcross, GA) to assess the size of the DNA fragments.

2.5 | Archaeal PCR, real-time quantitative PCR and DGGE

PCR and Denaturing Gradient Gel Electrophoresis (DGGE) were performed as described previously (Carbonero et al., 2012). Initial PCR was performed with the universal Archaea primers 1Af/1100r (annealing temperature: 55°C) to check for the presence of Archaea 16S rDNA gene sequences, then a nested PCR was performed on the initial PCR amplicons with the primers PA340f-GC/PA519r (annealing temperature: 63°C) (Carbonero et al., 2012). PCR reactions were performed following the same approach (hotstart [2 min at 96°C]; 30 cycles of denaturing for 1 min 30 s at 96°C , 2 min at the annealing temperature and elongation for 2 min at 72°C ; final elongation at 72°C , 5 min) in 25 μl reaction volumes using a GoFlexiTaq kit (Promega; 0.2pM primers, 1 μl template DNA). The final PCR products (20 μl) were analyzed on 8% (w/v) acrylamide/bis-acrylamide (37.5:1; 40% w/v) gels with a 20%–60% denaturant gradient. DGGE gels were run for 16 h at 75 V and at 60°C , stained with SybrSafe (Life Technologies), then visualized by UV transillumination.

The total archaeal community abundance was quantified as described previously (Carbonero et al., 2012; Carbonero et al., 2011;

Nava et al., 2012; O'Keefe et al., 2015). Briefly, archaeal 16S rDNA gene fragments (c. 140 bp) were amplified using universal primers Arch 967F and Arch-1060R using the SYBR® Green PCR Master Mix (Applied Biosystems) in 25 μ l reaction volumes (0.2 pM primers, 1 μ l template DNA). To estimate archaeal small-subunit rDNA gene abundances, standard curves were generated using a 10-fold serial dilution of *Methanosarcina acetivorans* genomic DNA.

2.6 | Dual-index bacterial amplicon library preparation and sequencing

A dual-index strategy previously described (Kozich et al., 2013) was followed to generate amplicon libraries of V3/V4 region of the 16S rDNA gene sequence. The PCR reactions were composed of 22 μ l of Pfx Supermix (Accuprime, USA), 1 μ l of each primer and 3 μ l of DNA template to reach a total volume of 27 μ l. The PCR consisted of an initial denaturation step at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 15 s, and elongation at 72°C for 5 min and a final elongation step (10 min at 72°C), on a MasterCycler thermocycler (Eppendorf). Several PCR products were chosen randomly and run on a 2% electrophoresis gel to verify the success of the amplification. Purification and normalization of PCR products were performed using the SequalPrep Normalization Plate (96) kit (Invitrogen, Carlsbad, CA).

The length of the amplicon fragments was analyzed on a TapeStation (Agilent Technologies, Santa Clara, CA) and the concentration of the amplicon pools was determined by quantitative PCR (qPCR) using the PerfeCta NGS Library Quantification kit for Illumina (Quanta Biosciences, Beverly, MA). The qPCR program consisted of an initial activation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s, using a Realplex materycycler (Eppendorf). A final melting curve was generated at the end of the program and the concentration was calculated using a standard curve.

The pool was then denatured with 0.2N NaOH and diluted to 12.5 pM with HT1 buffer. Twenty percent PhiX control was added to the denatured DNA. Note that 600 μ l of the pool and 3 μ l of each required sequencing primers were loaded into the respective wells of the Illumina Miseq reagent cartridge. Once the run was completed, the reads were downloaded from the Illumina Basespace server.

2.7 | Bioinformatics and statistical analyses

The analysis of the sequences was performed using Mothur version 1.39.5 (Schloss et al., 2009) using the SILVA database (Quast et al., 2013) as a reference. Nonmetric dimensional scaling (NMDS) and analysis of similarities (ANOSIM) based on Bray-Curtis index were performed to evaluate the microbial diversity of the products using PAST 3.15. The same software was used to calculate the Shannon diversity index. Significant differences of bacterial taxa between time points or

samples were detected using Analysis of Variance (ANOVA). Differences were considered significant when $p < 0.05$.

3 | RESULTS AND DISCUSSION

3.1 | Archaeal microbiota of different plant-based fermented food and beverages

Fermented crunchy kraut, jalapenos and okra were all positive for Archaea by conventional PCR. As expected, a modest diversity in Archaea phylotypes was observed, with four to nine DGGE bands per sample (data not shown). Quantitative PCR was used on PCR-positive samples and confirmed the widespread presence of low abundance archaeal populations. In the brine of fermented vegetables, archaeal gene copy numbers were all around 2000 per ml (Figure 1a). Kraut samples taken at different stages of fermentation confirmed that the brine harbored Archaea initially and importantly, their gene copy numbers increased steadily throughout fermentation, demonstrating that they were actively growing in the brine (Figure 1b).

To the best of our knowledge, this is the first report of Archaea growing in the brine of fermented vegetables produced in the United States of America. There is ample evidence from studies in Asian countries that halophilic Archaea are seeded from the salt added to the brine (Chang et al., 2008; Henriot et al., 2014; Lee, 2013; Roh et al., 2010). However, there was little evidence in the literature on the abundance and the activities of such Archaea. To our knowledge, the only other study reporting archaeal abundance investigated Kimchi and reported numbers in the same order of magnitude (10^4 gene copies per ml) (Park et al., 2009). Importantly, the archaea numbers increased during the fermentation, indicating their ability to multiply in the brine environment. These observations open avenues of research to determine how Archaea in these and other products may affect quality and chemical profiles.

3.2 | Surface bacterial microbiota profiles of unfermented vegetable and tea

The microbiota profiles as demonstrated by NMDS showed relatively consistent clustering by food type. Jalapenos and kraut formed narrow clusters relatively close together, while okra samples exhibited more interindividual variation and clustered apart from the other food samples (Figure 2a). The jalapenos samples had the highest number of bacterial taxa found with an average of 58 different genera per sample (Table 1). The cabbage, okra, and tea were very similar regarding the number of genera per sample; however, the tea samples showed an important internal variation. The Shannon indices indicated a higher diversity for the okra, followed by the kraut and finally the jalapenos.

Overall, the main phyla found in the four food products before fermentation were proteobacteria followed by firmicutes and Bacteroidetes (Figure 2b). The jalapenos samples were largely dominated by proteobacteria (93%), the two other phyla only accounted for 3%

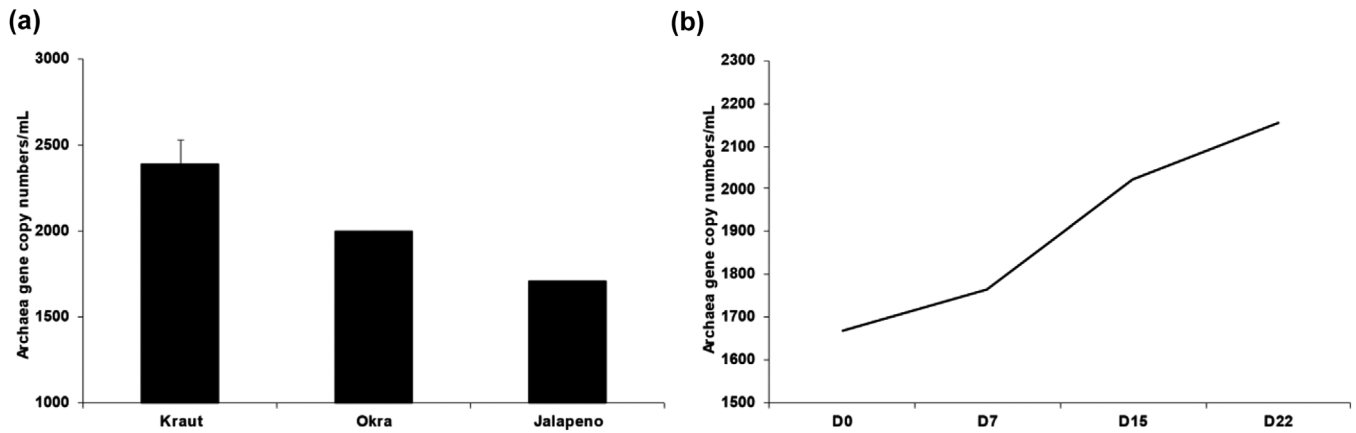


FIGURE 1 Abundance of Archaea 16S rDNA gene sequence in different food products. (A) Archaea gene copy numbers/mL in the brine of fully fermented crunchy kraut, okra, and jalapeno peppers. (b) Temporal increase of Archaea gene copy numbers from day 0 to day 22 of crunchy kraut fermentation

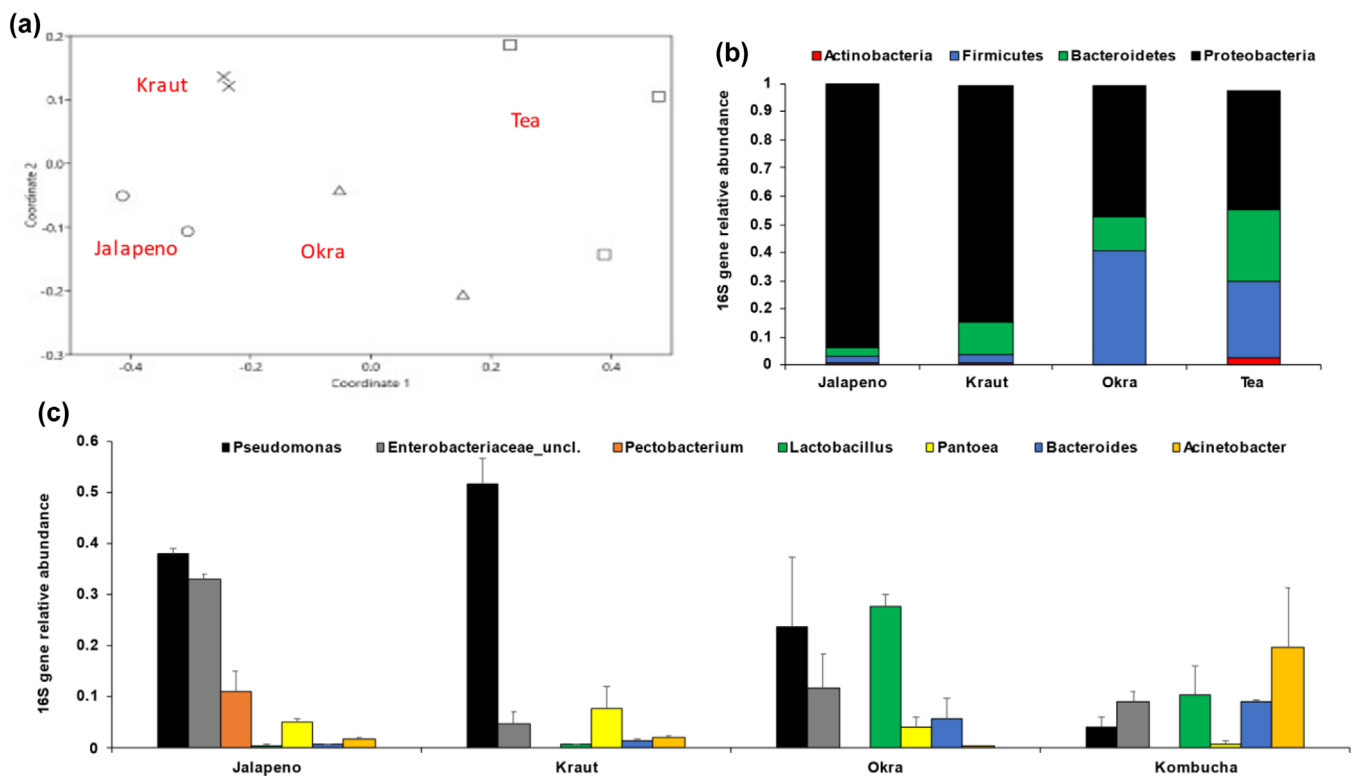


FIGURE 2 Comparison of the bacterial microbiota profiles of jalapenos, kraut, okra, and tea before fermentation (A) Nonmetric multidimensional scaling (NMDS; Bray–Curtis dissimilarity index) indicates significant differences (ANOSIM, $p < 0.05$) between each food product microbiota. (b) Phylum-level profiles of the unfermented food products. (c) Genus-level taxonomic profile highlight the specificities of each food product

each. The kraut samples show a similar distribution with a large majority of proteobacteria (84%), however Bacteroidetes were more abundant than firmicutes (11% and 3%, respectively). The three dominant phyla in the okra and tea samples were more evenly distributed. The most represented phylum was still proteobacteria (47%) in the okra but was closely followed by firmicutes (40%). The other phyla found in the vegetables accounted for less than 2% of the total sequences and were

mainly represented by Actinobacteria, and Verrucomicrobia. The tea samples contain more minority phyla; Acidobacteria, Actinobacteria, Chlamydiae, Synergistetes, and Verrucomicrobia represented a total of 5% of the number of bacteria.

The foodborne population found in the vegetables and tea products were dominated by, *Pseudomonas*, *Pantoea*, and other unclassified Enterobacteriaceae; *Lactobacillus* and *Bacteroides* (Figure 2c). The

TABLE 1 Number of taxa and diversity indices of the bacterial ecology of unfermented plant-based products (T0), along the fermentation (T7 and T14), at the end (TF) and after five days of cold storage (TF+5)

	Sauerkraut		Okra		Jalapenos		Kombucha		
	No. taxa (genera)	Shannon index (H')	No. taxa (genera)	Shannon index (H')	No. taxa (genera)	Shannon index (H')		No. taxa (genera)	Shannon index (H')
T0	47 (± 9)	2.13 (± 0.2)	46 (± 5)	2.19 (± 0.51)	58 (± 2)	1.78 (± 0.006)	Tea	42 (± 34)	2.75 (± 0.49)
T7	35 (± 10)	1.64 (± 0.02)	44 (± 4)	0.6 (± 0.06)	37 (± 2)	1.61 (± 0.04)	T0	37 (± 15)	2.49 (± 0.02)
T14	34 (± 18)	1.16 (± 0.3)	15 (± 4)	0.5 (± 0.14)	31 (± 6)	1.49 (± 0.11)	T1	48 (± 18)	2.45 (± 0.14)
TF	16 (± 1)	1.41 (± 0.04)	14 (± 2)	0.47 (± 0.08)	22 (± 1)	1.36 (± 0.05)	T3	32 (± 11)	2.64 (± 0.08)
TF+5	17 (± 2)	1.37 (± 0.03)	13 (± 1)	0.48 (± 0.06)	14 (± 3)	1.55 (± 0.03)	T7	26 (± 9)	2.37 (± 0.5)

kraut harbored a distinct bacterial microbiota with *Pseudomonas* representing more than 50% of the bacterial community, which is much more than previously reported for cabbage (Wassermann et al., 2017). It can be hypothesized that the root vegetables (carrots and Jicama) harbored relatively abundant soil-borne bacterial populations, including *Pseudomonas*, as has been described for potatoes (Köiv et al., 2015). Overall, the kraut microbiota was more similar to the potato than the cabbage as described previously, notably missing members of Burkholderiales and Lactobacillales. Strikingly, the unfermented kraut harbored extremely small amounts of LAB (*Lactobacillus* and *Leuconostoc* combined abundances close to 1%), indicating that starter culture is not a requirement for successful spontaneous lactic acid fermentation. Similarly, *Pediococcus* has been isolated several times on cabbage as one of the dominant LAB (Di Cagno et al., 2013) but was not detected in this study.

The jalapenos harbored a somewhat similar bacterial microbiota to the kraut mixed vegetables, with more Enterobacteriaceae and less *Pseudomonas*, presumably because there were less root vegetables included. *Pectobacterium* were detected in significantly higher levels than other vegetables and are known as crops pathogens (Waleron et al., 2014).

Okra studied here harbored significantly higher levels of *Lactobacillus* (28%) than any other products potentially indicating high prevalence on these plants. It appears that these high amounts of *Lactobacillus* resulted in competitive exclusion of Proteobacteria, and potentially for *Bacteroides* and *Prevotella* to develop larger populations (6% and 2%, respectively), which may result in okra pods being less prone to bacterial spoilage and human pathogens contamination. Indeed, mostly fungal leaf diseases are documented for this vegetable (Soliman & El-Mohamedy, 2017). However, product handling and preparation may also explain these trends, and okra from various sources should be tested to confirm this hypothesis.

In comparison with the vegetable products, the bacterial community found in the tea was more diverse with several genera represented but without any clear dominance. It is expected that the water used to brew tea is sterile after boiling; therefore, the bacterial populations must have been seeded by the tea leaves and possibly the containers. *Acinetobacter*, *Lactobacillus*, and *Bacteroides* were the dominant genera which are known to be plant-borne and present in the human microbiota (Thomas et al., 2011), therefore both environmental and human

sources are possible. Other Firmicutes in the tea comprised somewhat evenly distributed amounts of *Clostridium spp.* and Ruminococcaceae, which may also have human origins (Flint et al., 2012).

Other bacterial genera have been found in the samples with a lower abundance. *Yersinia* was detected in the okra, jalapenos, and the kraut. However, only the okra samples showed a significant difference with the tea for this genus ($p = 0.037$). This genus is present in the environment but also contains several human pathogens (McNally et al., 2016). *Acinetobacter* was detected in all foods but was more abundant in the tea samples and had previously been extracted from soil and water (Baumann, 1968). Other environmental Proteobacteria were detected, mainly from the kraut. *Leuconostoc* are considered part of the autochthonous LAB that can be isolated on raw vegetables and plants and can initiate fermentation, in particular sauerkraut when the conditions are favorable (Di Cagno et al., 2013). Here, only okra was found to harbor relatively high *Leuconostoc* levels (3%).

3.3 | Bacterial microbiota dynamics of fermented crunchy kraut

According to NMDS and the one-way ANOSIM, the kraut samples at T0 and T7 were distinct from each other and statistically different from T14 and the latter samples (Supporting Information Figure S1a). Five phyla were detected in the kraut (and the other vegetables) samples: Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Verrucomicrobia, but only the first three accounted for more than 1% for at least one time point, and Bacteroidetes fell below 1% at all time-points (Figure 3a). The Proteobacteria phylum was the most abundant at the beginning of the fermentation (84%) and decreased during the first two weeks of the process, to be significantly lower at T14. Firmicutes, which were not predominantly represented in the fresh mix, had a low abundance at the start of the fermentation (4%) and steadily increased until T14. Sauerkraut and other cabbage fermentation are well known to be dominantly performed by Firmicutes (Di Cagno et al., 2013; Kyung et al., 2015), however it is more surprising to observe maintenance of significant levels of Enterobacteriaceae over a few days. The number of taxa decreased drastically during fermentation (Table 1), which probably reflects death of several genera outcompeted by dominant fermenters. The Shannon index shows a

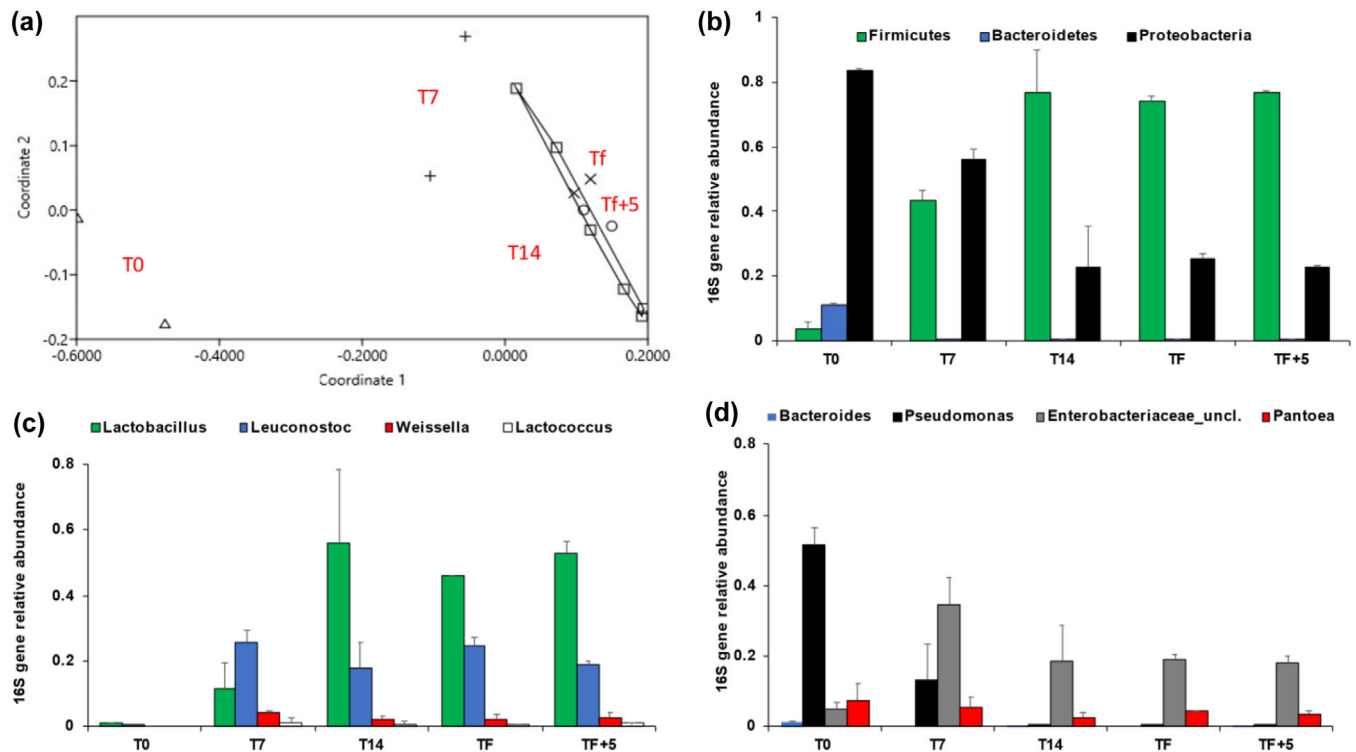


FIGURE 3 Bacterial successions along crunchy kraut fermentation. (A) NMDS representation of the bacterial dynamics (triangles are for T0, plus sign for T7, squares for T14, x sign for TF and circles for TF+5). (B) Phylum level dynamics indicate three distinct stages at T0, T7, and a final stage from T14 until the end of the fermentation (TF: end of fermentation, and TF+5, after five days of cold storage). (C) Lower level taxonomic profiles indicate succession of *Leuconostoc* then *Lactobacillus* as major Firmicutes fermenters, while (D) indicates maintenance of relatively high levels of *Enterobacteriaceae* and *Pantoea*

diminution of diversity in the samples overall, confirming the number of taxa results.

As reported for other cabbage fermentation, the dominant Firmicutes were *Lactobacillus* and *Leuconostoc*; however, *Weissella* and *Lactococcus* that were undetected on unfermented materials also increased to levels between 1% and 5% (Figure 3b). At day 7, *Leuconostoc* were the dominant Firmicutes, and their abundance was maintained over the fermentation while *Lactobacillus* became the largely dominant population at day 14. *Lactobacillus* and *Leuconostoc* and their dynamics are known as the driver in the succession of the heterofermentative and homofermentative stages of the lactic acid fermentation (Di Cagno et al., 2013). *Lactococcus* have been used as a starter culture for sauerkraut fermentation (Song et al., 2017), however their abundance was still stimulated in sauerkraut produced without starter culture. *Weissella* have been described as plant-associated and present spontaneously in different food fermentation (sourdough and African and Asian traditional fermented foods) (Fusco et al., 2015) *Weissella* species have also been recovered in the first week of commercial sauerkraut fermentation (Plengvidhya et al., 2007). Those latter genera have been described in less controlled cabbage fermentation and other vegetables (radish, mustard) fermentation; therefore, it can be hypothesized that the presence of other vegetables increased Firmicutes diversity.

The Proteobacteria were initially dominated by soil-borne *Pseudomonas*, however, these were rapidly taken over by unclassified Enter-

obacteriaceae (Figure 3c). At lower abundances, *Pantoea* maintained relatively stable abundance. Their persistence may be explained by the capability of some of their species to be resistant to acidity, since *Pantoea agglomerans* can form a biofilm and synthesize a protein providing it acid-resistant properties (Li et al., 2012). It is also likely that Proteobacteria populations actually decreased as expected, but Proteobacteria DNA sequences were still picked up, and overestimated to some extent by the microbiome approach that cannot distinguish between actually growing and dying or even dead cells.

3.4 | Bacterial microbiota dynamics of fermented jalapenos and okra

According to NMDS and the one-way ANOSIM, the jalapenos samples at T0 and T7 were distinct from each other and statistically different from T14 and the latter samples (Supporting Information Figure S1b). In contrast, only the T0 samples were different from any other time points for the okra (Supporting Information S1c). The different dynamics were due to the different original microbiota. Jalapenos dynamics were comparable to the kraut dynamics at the phylum level (Figure 4a), while the low levels of Proteobacteria in okra resulted in rapid bloom of Firmicutes reaching around 90% (Figure 4b). Absence of competition from Proteobacteria is a desirable trait, and it is possible that okra

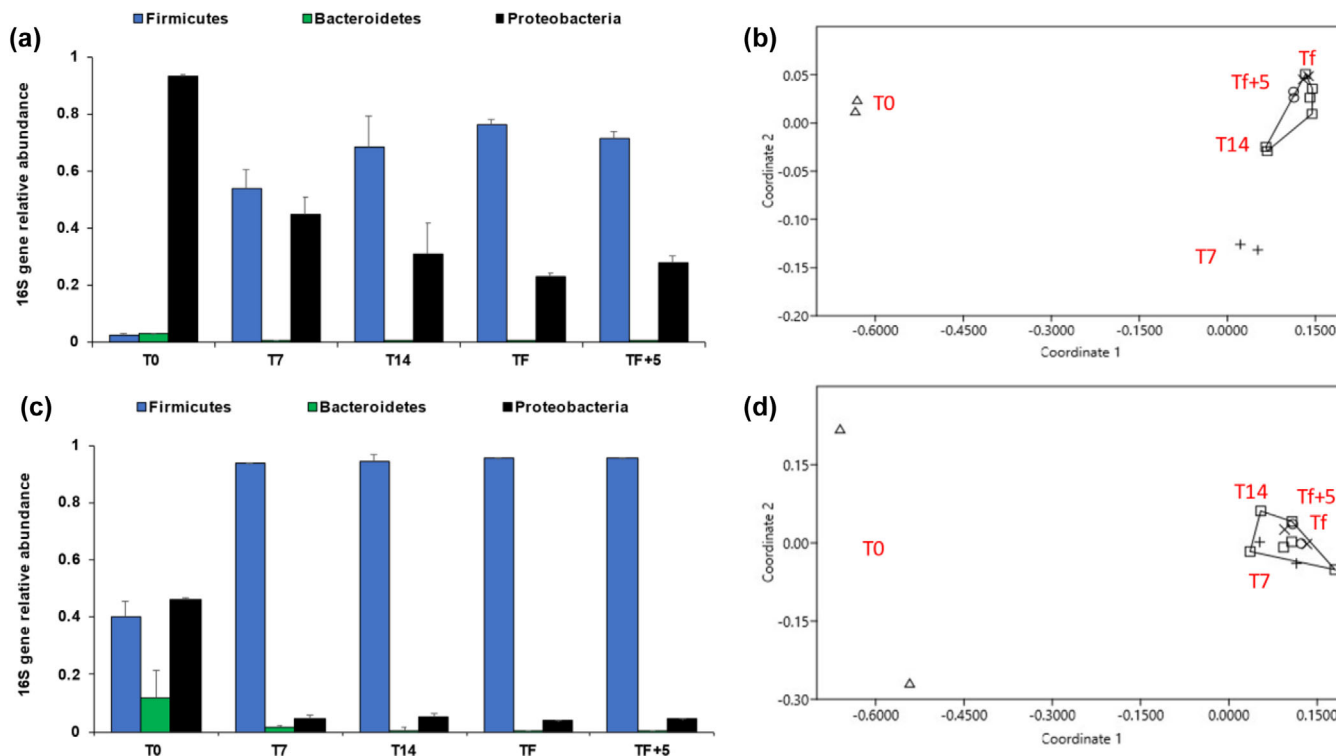


FIGURE 4 Bacterial successions along jalapenos (A and B) and okra (C and D) fermentation. (A and C) Phylum level dynamics and (B and D) NMDS indicate three stages (initial, T7 and final) for jalapenos (A and B) and two stages (initial T0 and final) for okra (C and D)

fermentation could be completed in a shorter timeframe. The number of taxa during the fermentation of the jalapenos decreased throughout fermentation, indicating the loss of several genera, potentially outgrown by the remaining ones. The Shannon index decreased during the fermentation but increased again during the storage (Table 1).

For the jalapenos, the Firmicutes and Proteobacteria genera dynamics were almost identical to the kraut dynamics (Figure 5a and b). Of note, *Weissella* and *Lactococcus* reached higher abundances than in the kraut. These similarities may be explained by the fact that plant materials were shredded like the cabbage, allowing for unrestricted access to all the different nutrients. It has been previously shown that fermentation of whole-head cabbage and shredded cabbage showed a slower growth of LAB in the whole product, but they reached a higher population for a longer time than in the shredded sauerkraut. A slower diffusion of nutrients during the fermentation of the whole-head cabbage resulted in a slower production of acid and a longer survival of the bacteria (Niksic et al., 2005). *Weissella*, *Lactobacillus*, and *Leuconostoc* were previously described as the main LAB present during the fermentation of peppers (González-Quijano et al., 2014). Low abundant Proteobacteria were more specific of the jalapenos, with maintenance, albeit at levels just below 1%, of *Pectobacterium*, which appeared to have been seeded from the plant materials (Figure 5b). Interestingly, a different potential pathogenic genus, *Cronobacter*, was undetected in raw materials but appeared at day 7 and maintained populations just above 1% throughout the fermentation. *Cronobacter* species have been found in the environment and associated with plants, but can also lead to human infections (Joseph & Forsythe, 2012).

In this study, okra fermentation dynamics were completely distinct than kraut and jalapenos dynamics (Figure 5c and d). Okra fermentation was almost exclusively performed by *Lactobacillus* species, which were presumably seeded from raw materials (29%). There have been several reports that olives' fermentation is primarily performed by foodborne *Lactobacillus*, leading to claims of probiotic properties (Montoro et al., 2016). These similarities in microbiota dynamics may come from the food presentation (whole rather than shredded) that determines the rate of diffusions of nutrients available in the brine and therefore influence the growth of the lactic acid producing bacteria (Niketić-Aleksić et al., 1973; Niksic et al., 2005).

3.5 | Bacterial microbiota dynamics of kombucha

From the results obtained, it is difficult to delineate specific bacterial successions for kombucha, especially given that the two batches that were started from the same SCOBY only one week apart did not cluster together in the NMDS (Supporting Information Figure S1d). Essentially, most fermentation stages cluster apart from nonfermented tea. There is a trend for final fermentation stages to cluster apart, but somewhat close to nonfermented samples. Four phyla were detected in both batches: Proteobacteria, Bacteroidetes, Firmicutes (Figure 6a), and Fusobacteria at very low levels. There was no consistent trend for Firmicutes and Proteobacteria that fluctuated in abundances, but in both batches Bacteroidetes significantly increased at the end of the fermentation. Fusobacteria were not detected in the tea but appeared

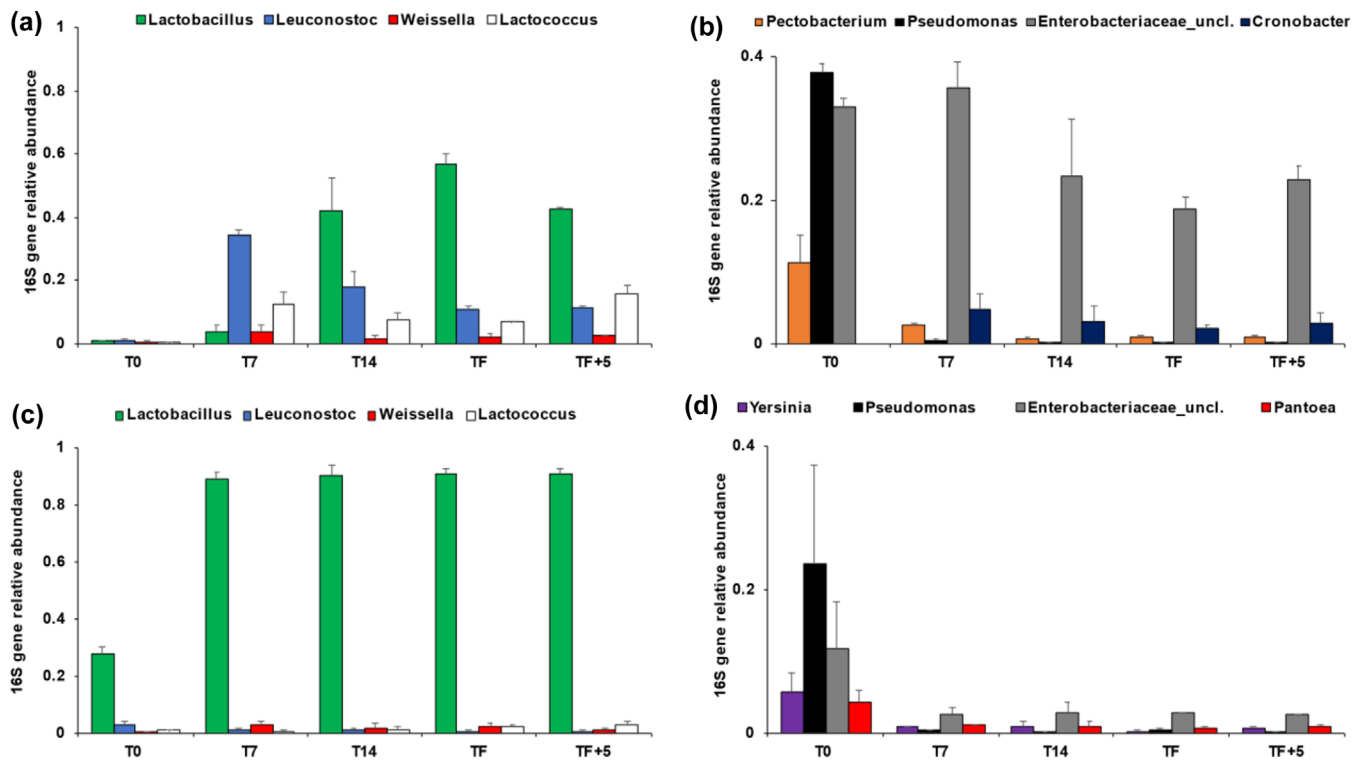


FIGURE 5 Genus-level bacterial succession. Profiles indicate increasing Firmicutes members during Jalapeno (A) and okra fermentation (C). Lower-level taxonomic profiles indicate a largely *Lactobacillus* driven fermentation for okra. The diversity and abundance of Proteobacteria members decreased during the fermentation of both products (B and Df)

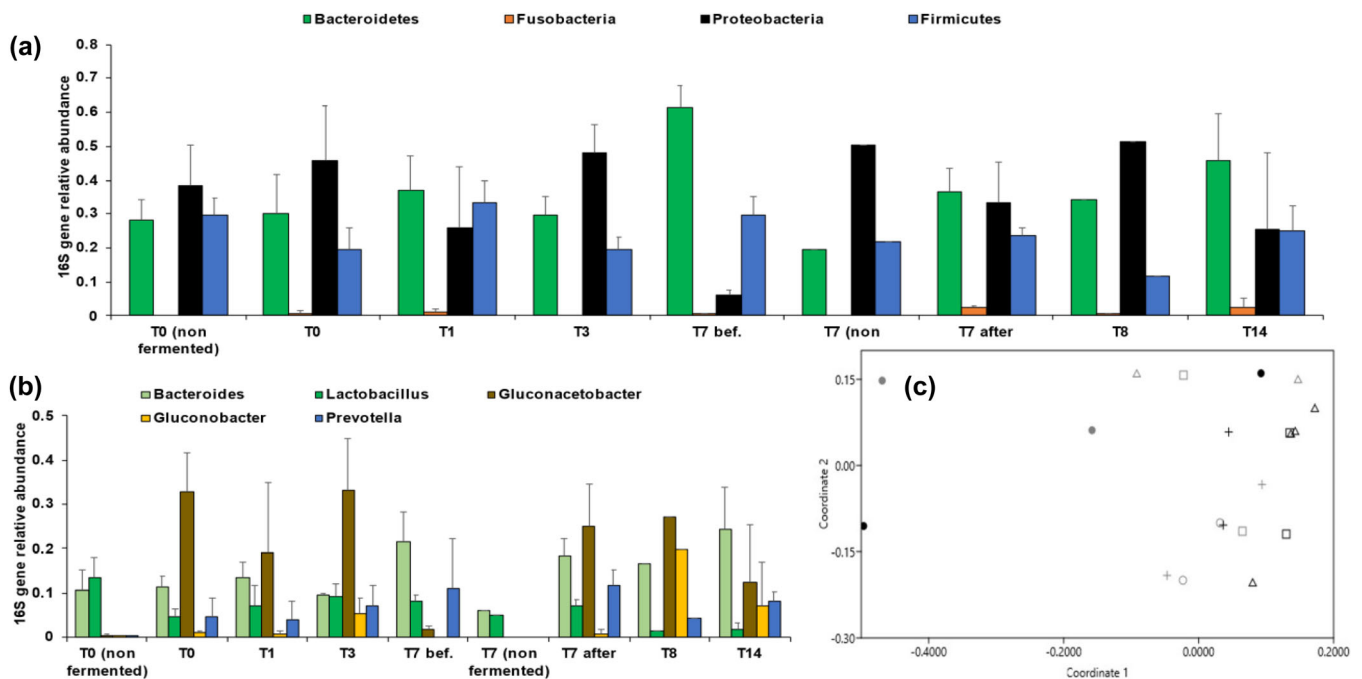


FIGURE 6 Bacterial successions along kombucha fermentations. (A) Phylum level dynamics indicate no consistent trends between the two fermentation batches. (B) Lower-level taxonomic profiles indicate a dominance of *Gluonacetobacter* and *Bacteroides* during fermentation. (C) NMDS representation of the bacterial dynamics during the fermentation kombucha (For the kombucha, dot is for non-fermented tea, plus signs for T0, square for T1, circle for T3 and triangles for T7; Gray symbols: week 1; Black symbols: week 2)

during the fermentation, suggesting contamination from handlers (Allen-Vercoe et al., 2011) or the vessel. Less abundant phyla were also found during the first week, Chlamydiae, Synergistetes, and Verrucomicrobia in all samples, all under 1.5% at most. They were not found in the second batch; however, sequences belonging to Actinobacteria were more abundant than during the first week. All those phyla may originate from the environment and are unlikely to participate in the fermentation except for Actinobacteria. Several external and internal parameters (oxygen level, pH, temperature, nutrient availability) may explain the lack of interbatch convergence in bacterial successions.

Richness and diversity generally decreased slightly during fermentation, with large variation between batches (Table 1). In contrast with the vegetables, several genera from the three dominant phyla appeared involved in kombucha fermentation: *Gluconacetobacter*, *Bacteroides*, and *Lactobacillus*, and less consistently *Gluconobacter*, *Prevotella*, and other Ruminococcaceae and Acidaminococcaeae (Figure 6b). This observation contrasts with previous reports of almost exclusive dominance of *Gluconacetobacter* (Chakravorty et al., 2016; Coton et al., 2017; Marsh et al., 2014; Podolich et al., 2017), or its association with *Oenococcus* in green tea based kombucha (Coton et al., 2017). Among Proteobacteria, Enterobacteriaceae and Acinetobacter which were prevalent in the tea, decreased significantly in relative abundance during fermentation. However, since bacterial populations should have been limited in size in the tea, it is likely that they actually maintained low numbers all along the process. *Gluconacetobacter* and *Gluconobacter* in the second batch increased rapidly and decreased at the end of fermentation. These two genera perform acetic acid fermentation commonly reported in kombucha (Chakravorty et al., 2016; Coton et al., 2017; Marsh et al., 2014), however high acetic acid is generally considered undesirable for the final taste. Because *Gluconacetobacter* and *Gluconobacter* levels were lower than other kombucha produced in Europe (Coton et al., 2017), it can be hypothesized that SCOBY are selected according to local consumer taste. Among Firmicutes, *Lactobacillus* were dominant in the first batch but in lower concentration, while a range of other taxa were also detected throughout the fermentation (*Clostridium*, Ruminococcaceae, Lachnospiraceae, all below 10%). *Lactobacillus* release lactic acid and a typical sour taste, while the other Firmicutes mostly release short-chain fatty acids that can develop off-odors at low levels and off-taste at higher levels. In addition, *Lactobacillus* species have been reported to be able to metabolize polyphenols from tea (Theilmann et al., 2017). Again, the differences in the main Firmicutes fermenters will result in different taste, with the kombucha studied here being sourer than other kombucha studied in Europe (Coton et al., 2017). The most remarkable genera detected in these kombucha samples are *Bacteroides* and *Prevotella* (Figure 6b). Both are known as dominant human gut microbiota members, with distinct affinities for carbohydrates and proteins (O'Keefe et al., 2015). Neither has previously been reported as dominant fermenting bacteria in fermented foods, while during the fermentation of these kombucha samples, their combined relative abundance is always at least over 15%. Both genera release short-chain fatty acids, in particular propionic acid (Schwiertz et al., 2010), which would have limited impact on the kombucha taste. *Bacteroides* species have been reported

to metabolize polyphenols from different plant sources (Sheflin et al., 2017). However, in vivo studies have reported tea and its polyphenols to inhibit the growth of *Bacteroides* and *Prevotella* in human or murine gut microbiota (Duda-Chodak, 2012). Since *Bacteroides* reach their highest abundance at the end of fermentation, it is possible that they further metabolize polyphenols by-products from *Lactobacillus* at this time, while *Lactobacillus* and *Prevotella* mainly ferment polysaccharides in the early stages of fermentation.

3.6 | In vitro fermentation

According to the nonmetric MDS plot (Supporting Information Figure S2), the composition of the batch cultures evolved steadily along the simulated fermentation, and all samples clustered by time points, except for the samples treated with the kraut brine. However, starting at day 4, the kombucha and kombucha supernatant treatment tended to cluster apart from the kraut and kombucha pellet samples. Since the pellet samples were nutrient poor, it can be hypothesized that nutrients derived from microbial cell death are utilized by the bacteria from the stool sample. The composition of the samples is significantly different for every time point except between T4 and T12 (one-way ANOSIM test). However, no difference is shown between the treatments. The only one seeming to evolve distinctly from the others is the kraut brine but the difference was not significant (p value = 0.416).

Five phyla were recovered from the samples (Figure 7a). The most abundant initially was Bacteroidetes, followed by Firmicutes and Proteobacteria. Fusobacteria and Actinobacteria were present in lower concentration. Over time, the population of Bacteroidetes decreased while the Firmicutes increased. The population of Proteobacteria stayed the same for the samples treated with kombucha but decreased by 22%–29% in the kraut treatments. Actinobacteria increased only in the samples treated with kombucha and particularly the kombucha supernatant.

The in vitro fermentation bacterial microbiota included genera typically detected in the human colon; with significantly lower richness and diversity (Faith et al., 2013). This is a well-documented limitation of in vitro stool fermentation experiments (Aguirre & Venema, 2017). *Bacteroides* was the dominant genus and represented between 40% and 50% of the sequences in the samples, with an overall decreasing trend in all in vitro fermentations. The abundance of *Megamonas* increased in all samples, and more markedly in samples containing more nutrients (kombucha and supernatant samples). In particular, *Megamonas* reaching 13%–24% relative abundance in the kraut brine fermentation drove the distinct clustering in the NMDS. *Megamonas* are irregularly detected in human gut microbiota and generally at low levels (Kuang et al., 2016), and their metabolic role is not well known.

Probiotics and other beneficial genera were studied, with emphasis on genera with polyphenol metabolizing abilities, since tea and kombucha are known to contain substantial levels of polyphenols. Specifically, epicatechins and potentially antioxidant metabolites were shown to increase after kombucha fermentation, as a result of the metabolic activities of microorganisms (Jayabalan et al., 2007; Jayabalan et al.,

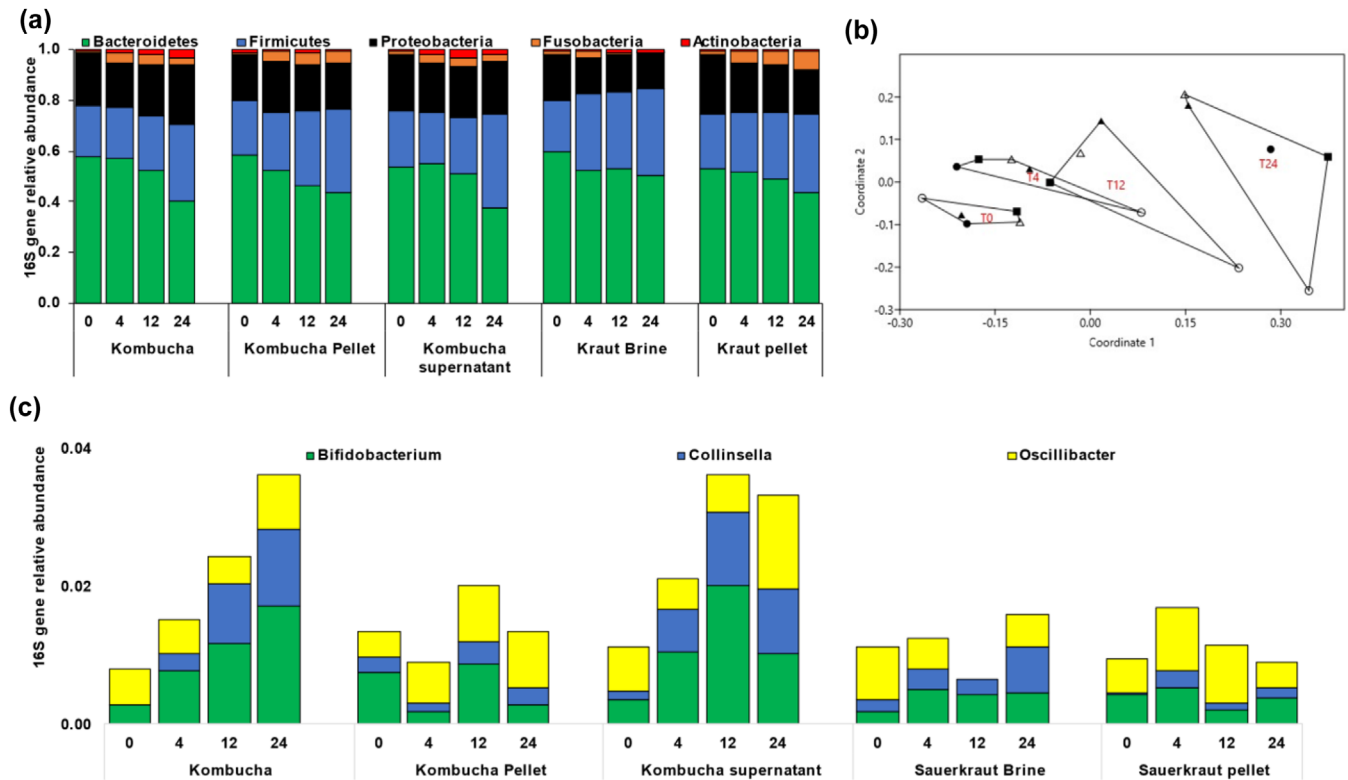


FIGURE 7 In vitro gut microbiota fermentation after 0, 4, 12, and 24 h of fermentation. (A) phylum-level dynamics are characterized by a gradual decrease in abundance of Bacteroidetes (more abundant initially) and gradual increase of Firmicutes (more abundant in final time-points). (B) Nonmetric multidimensional scaling (NMS; Bray-curtis dissimilarity index) of the stool microbiota dynamics over the in vitro fermentation experiments: dots: kombucha; filled triangles: kombucha pellet; filled squares: kombucha supernatant; circles: kraut brine; triangles: kraut pellet (C) Lower-level taxonomic profile of genera of interest

2008). *Lactobacillus* were expected to be present since they were abundant in kraut and kombucha samples, and they are known as primary polyphenols metabolizers (Sheflin et al., 2017); however, they failed to establish themselves in any experiment. No significant trend with any genus was observed in the kraut samples and the kombucha pellet sample. However, the kombucha and especially the kombucha supernatant were found to significantly stimulate the growth of *Bifidobacterium*, *Collinsella*, and *Oscillibacter* (Figure 7b). *Bifidobacterium* and *Collinsella* are Actinobacteria members both known to be involved in polyphenol degradation (Guadamuro et al., 2017; Jaquet et al., 2009; Li et al., 2015; Mills et al., 2015). Here, their stimulation suggests that kombucha provides prebiotic (and not probiotic as often claimed) properties, potentially by breaking down native polyphenols present in tea to intermediate metabolites. *Oscillibacter* has been reported as beneficial (Li et al., 2016), and potentially involved in polyphenol metabolism (Anhê et al., 2018; Xie et al., 2017).

4 | CONCLUSIONS

Overall, our results indicate that fermented vegetables are likely to harbor a low abundant but apparently diverse Archaea community. While the impact on food properties is expected to be marginal, a bet-

ter characterization of fermented food Archaea is warranted, as well as testing other food types for presence of viable Archaea.

While raw vegetables were found to harbor plant-associated bacteria as expected, brewed tea surprisingly harbored a distinct bacterial microbiota. Shredded kraut and jalapenos fermentation dynamics followed the classic lactic acid fermentation, while okra fermented as whole exhibited fermentation dynamics more comparable to that of olives. The kombucha dynamics in this study were very different from other kombucha fermentation described previously, indicating that both SCOBY and tea original microbiota influence the fermentation. The most intriguing finding was that probiotic potential was not supported by in vitro experiment, but rather a significant potential prebiotic effect from kombucha.

ACKNOWLEDGMENTS

The authors would like to thank Drs Daniel and Faith Lessner for providing *Methanosarcina acetivorans* genomic DNA.

This work was supported by the Department of Food Science and the Dale Bumpers College of Agricultural, Food and Life Sciences at the University of Arkansas.

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How to cite this article: Lavefve, L., Cureau, N., Rodhouse, L., Marasini, D., Walker, L. M., Ashley, D., Lee, S.-Ok, Gadonna-Widehem, P., Anton, P. M., Carbonero, F. (2021). Microbiota profiles and dynamics in fermented plant-based products and preliminary assessment of their in vitro gut microbiota modulation. *Food Frontiers*, 1–14. <https://doi.org/10.1002/fft2.75>