

1 **Investigation of the Lactic Acid Bacteria in Sauerkraut Fermentations by DNA**
2 **fingerprinting**

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15 Running head: Microbiota of sauerkraut

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ABSTRACT

Previous studies using traditional biochemical identification methods to study the ecology of commercial sauerkraut fermentations revealed that four lactic acid bacteria species, *Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Lactobacillus brevis* were the primary microorganisms in the fermentation. In this study 686 isolates were collected for analysis by DNA fingerprinting, from four commercial fermentations. The results indicate that the species of lactic acid bacteria present in sauerkraut fermentations are more diverse than previously reported, including *Leuconostoc citreum*, *Leuconostoc argentinum*, *Lactobacillus paraplantarum*, *Lactobacillus coryniformis*, and *Weissella* sp. The newly identified species, *Leuconostoc fallax* was also found. Unexpectedly, only 2 isolates of *Pediococcus pentosaceus*, and 15 isolates of *Lactobacillus brevis* were recovered during this study. A better understanding of the microbiota may aid the development of low salt fermentations, which may have altered microflora and altered sensory characteristics.

Keywords: sauerkraut, fermentation, lactic acid bacteria, PCR, microbial diversity

INTRODUCTION

42
43 Sauerkraut fermentation involves many physical, chemical and microbiological changes
44 that influence quality and safety of the product. The fermentation can be broadly categorized as
45 having successive stages, including an initial heterofermentative stage, followed by a
46 homofermentative stage (11, 23). Historically, four species of lactic acid bacteria have been
47 identified as present in sauerkraut fermentations: *Leuconostoc mesenteroides*, *Lactobacillus*
48 *brevis*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*. The identification of these
49 microorganisms has been based on morphological and biochemical criteria (22). Several species
50 of lactic acid bacteria (LAB), other than the four species mentioned above, have been found in
51 cabbage fermentations, including *Lactobacillus curvatus*, *Lactobacillus sake*, *Lactococcus lactis*
52 subsp. *lactis*, and *Leuconostoc fallax* (3, 19, 25). Recently, six *L. fallax* strains have been isolated
53 from brine samples obtained from sauerkraut fermentations (2). The taxonomic characterization
54 of LAB has been modified, and new species have been described using molecular techniques (1,
55 10, 20, 24) . Improvements in molecular identification techniques for the study of microbial
56 ecology have created new opportunities for the analysis of food fermentations.

57 This study was carried out because of the need to reduce sodium chloride (salt) waste
58 from commercial vegetable fermentations. It is well documented that the concentration of salt
59 has a controlling influence on the microbial succession in a typical sauerkraut fermentation (11,
60 12, 22) . It may be possible to reduce salt waste by fermenting cabbage with 1% instead of the
61 typical 2% salt concentration. The introduction of a *L. mesenteroides* starter culture to the
62 fermentation could help ensure that the initial stage of the fermentation produces the desirable
63 flavor compounds (11). A method has been developed (23) to determine the ability of an
64 unmarked starter culture predominate over the indigenous microbiota in sauerkraut

65 fermentations. In that study (23) however, the effect of the starter culture on the indigenous
66 microbiota was not determined.

67 During the sauerkraut fermentation there is a rapid turnover of LAB species. The
68 dominant species present in the fermentation shifts within two to three days from less acid-
69 tolerant heterolactic LAB species to more acid-tolerant homolactic fermenting LAB species, with
70 sequential populations each reaching 10^8 to 10^9 CFU/g (11). Under normal conditions, the
71 fermentation is essentially complete within two weeks, with the most acid-tolerant species *L.*
72 *plantarum* predominating. Our objective was to characterize the dominant LAB species in the
73 successive stages of fermentation. There is strong evidence that the genetic diversity of many
74 ecosystems, as assessed by molecular techniques, exceeds the microbial diversity determined by
75 traditional culture based identification methods (26). However, due to the rapid succession of
76 LAB in sauerkraut, and the potential impact of large numbers of dead cells on culture-free
77 nucleic acid-based methods, we carried out this study using bacterial isolates. In this report we
78 characterized isolates from the microbiota of commercial sauerkraut fermentations using a rDNA
79 intergenic spacer PCR method (ITS-PCR) with a database of known ITS-PCR patterns for LAB
80 (4, and this report), supplemented with 16S rDNA sequence analysis. Several species of LAB
81 not previously found in sauerkraut were observed. Notably, *Weissella* and *Leuconostoc citrium*
82 species were found in the heterolactic phase of the fermentations, and two (*P. pentosaceus* and *L.*
83 *brevis*) of the four LAB species expected to be present were apparently minor constituents of the
84 microbiota. A better understanding of the microbial ecology of sauerkraut fermentations may aid
85 in the development of low salt fermentation technology, which may alter normal microflora and
86 sauerkraut flavor (Breidt and coworkers, unpublished).

87

88 MATERIALS AND METHODS

89 **Bacterial strains:** Bacterial strains were obtained from the Food Fermentation Culture
90 Collection, US Department of Agriculture, Agriculture Research Service, Raleigh, NC (Suppl.
91 Table 1), or isolated as described below. LAB were maintained at -80°C in MRS broth (Difco
92 Laboratories) supplemented with 16% glycerol, and grown on MRS broth or agar plates (MRS
93 broth supplemented with 1.5% agar, Difco). To inhibit aerobic yeast or mold growth on MRS
94 agar plate with sauerkraut isolates, MRS agar was supplemented with 0.2% sodium azide (Sigma
95 Chemical Co., St. Louis, MO), or plates were incubated in an anaerobic hood (Coy Laboratories,
96 Detroit, MI).

97 **Commercial sauerkraut fermentation and sample collection:** Sauerkraut
98 fermentations at a commercial processing plant in Wisconsin consisting of approximately 100
99 megagrams of cabbage were sampled for this study, including one tank in the first year of the
100 study (Y1), and three different tanks in the second year (Y2). Only one sauerkraut production
101 facility in Wisconsin was used in this study, because there are currently very few commercial
102 production facilities in the US. The fermentation tanks were of cement construction, with the
103 approximate dimensions: 5 m length by 5 m width by 4 m deep. Each fermentation was carried
104 out with approximately 2.3 % NaCl (final equilibrated concentration), which was added by a dry
105 salting process using shredded cabbage from mixed cultivars. The cabbage was manually spread
106 in the tanks, covered with plastic sheeting, and initially weighted down with water on top of the
107 plastic sheeting. The fermentation temperature was not controlled, but the temperature in these
108 commercial fermentations typically averaged 18°C . For analysis of cabbage prior to
109 fermentation, shredded cabbage samples (approximately 500 g) were collected in sterile plastic
110 bags prior to salting. Brine samples (100 ml each) from the fermentations were obtained for

111 microbial and biochemical analysis with a one-cm-diameter, perforated stainless steel tube that
112 was sealed at the bottom, from a depth of approximately 60 cm from the tops of the fermentation
113 tanks, and about 60 cm from the sides. The sampling apparatus was sanitized with dilute (10%)
114 Clorox solution and rinsed with sterile water prior to use. Brine and cabbage samples were
115 obtained on days 1, 3, 7, and 14 after the start of fermentation and were placed in 2 x 50 ml
116 sterile plastic tubes (#430829 Corning Inc., Corning, NY). The cabbage and brine samples were
117 transported to the laboratory by overnight mail in insulated boxes containing wet-ice packs to
118 maintain a temperature between 0 and 5°C. Samples were processed immediately upon arrival.

119 **Chemical and microbial analysis of cabbage samples.** For chemical and
120 microbiological analysis, 100 g of the shredded cabbage was blended in sterile glass blender jars
121 with 200 g of water for 3 min in a Waring blender (Waring Products, Torrington, CT) and then
122 homogenized in a stomacher (Stomacher 400, Tekmar, Cincinnati, OH) for 3 min at the
123 maximum speed using bags containing an internal filter. Filtrate from the extract, approximately
124 30 mL, was transferred to a 50-mL sterile plastic tube and frozen at -20°C for subsequent
125 chemical analysis. Prior to freezing, one mL of the cabbage extract was removed for
126 microbiological analysis (see below). Chemical analysis was carried out using high-performance
127 liquid chromatography. Organic acids and ethanol were analyzed using an anion-exchange
128 column (Aminex HPX-87H, Bio-Rad Laboratories) with a 0.8 mL/min flow rate of 0.03 N
129 H₂SO₄ at 75°C. A UV detector (UV-6000, Thermo Separation Products Inc., San Jose, CA) and a
130 differential refractometer (Waters 410, Waters, Milford, MA) were connected in series for
131 detection of organic acids (at 210 nm) and ethanol. Sugars and mannitol were separated by a
132 CarboPac PA1 column (Dionex Corp., Sunnyvale, CA) with a 0.8 mL/min flow rate of 0.12 N
133 NaOH at room temperature, and detected by a pulsed amperometric detector (model PAD-2;

134 Dionex). The salt (NaCl) content in brine was determined by titration with AgNO₃ solution using
135 4'5'-dichlorofluorescein as the indicator (13).

136 For microbial analysis, samples were diluted in sterile saline (0.85% NaCl) and plated on:
137 plate count agar (PCA, Difco Laboratories, Detroit, MI), modified violet red bile agar VRBG
138 (VRB agar, Difco, supplemented with 1% glucose), modified De Man, Rogosa and Sharpe
139 (MMRS) agar (MRS, Difco, supplemented with 0.2% sodium azide), and yeast extract and malt
140 agar (YM, Difco), containing chlortetracycline and chloramphenicol at 250 mg/L each, (Sigma
141 Aldrich, St. Louis, MO), to enumerate total aerobic microbiota, *Enterobacteriaceae*, LAB, yeasts
142 and molds, respectively. In addition, each brine sample was plated on unmodified MRS agar
143 (without sodium azide) for collection of LAB isolates. Plating and plate counting were
144 performed using a spiral plater (Model 4000, Spiral Biotech, Norwood, MA) with an automated
145 colony counter (Protos Plus, Microbiology International, Frederick, MD). From each of the five
146 sampling times (days 1, 3, 5, 7, and 14 after the start of fermentation) in Y1, 96 isolated colonies
147 from MRS agar were randomly selected and isolated on MRS agar, then cells were frozen in
148 MRS broth containing 16% glycerol at -80°C. For the four sampling times in Y2 (days 1, 3, 7,
149 and 14), 20 isolates were similarly obtained from each of three fermentation tanks, resulting in a
150 combined total for both years of the study of 720 possible isolates, although only 686 isolates
151 were recovered. The isolates were screened for gas production using Durham tubes (6 x 50 mm,
152 Kimble) inverted in 5 ml MRS broth. In addition, cells were cultured on homolactic-heterolactic
153 differentiation (HHD) medium (17).

154 **DNA extraction and PCR amplification:** MRS broth cultures from each fermentation
155 isolate were incubated at 30°C 12-16 h and then subjected to DNA extraction. Genomic DNA
156 was isolated using a Wizard™ Genomic DNA Purification kit (Promega Corporation, Madison,

157 WI) in accordance with the manufacturer's instructions, with minor modification. Twenty
158 microliters of mutanolysin (2.4 mg/ml, Sigma-Aldrich) was substituted for lysostaphin. The
159 method of Breidt and Fleming (4) was used to amplify the intergenic transcribed spacer (ITS)
160 region between 16S and 23S rRNA genes. Each 100 μ l of reaction mixture consisted of 10 μ l of
161 10X PCR buffer (500mM KCl and 100mM Tris-Cl, pH 8.0), 10 μ l of 25 mM MgCl₂, 1 μ l of
162 dNTP mixture (25mM each dNTP, Stratagene), 4 μ l of DNA preparation as described above, 70
163 μ l of water, 1 μ l of *Taq* DNA polymerase (5 U/ μ l), and 2 μ l of each primer. The primers used for
164 PCR amplification (4) were GAAGTCGTAACAAGG and GGGTTTCCCCATTCGGA. All
165 primers in this report were obtained from Sigma-Genosys (Sigma-Aldrich, St. Louis, MO). PCR
166 reactions were carried out using a model GTC-2 Genetic Thermal Cycler with a model LTM-2
167 refrigeration unit (Precision Scientific Inc., Chicago, IL). The temperature program consisted of
168 an initial heat denaturation step at 94°C for 5 min, 25 cycles of 1 min at 94°C, 5 min at 55°C,
169 and 2 min at 72°C followed by a final 5 min interval at 72°C. DNA products from the PCR
170 reaction were treated (without purification) with 1 μ l of *Rsa* I enzyme solution (16 U/ μ l. No.
171 500890, Stratagene, La Jolla, CA) for 1 h at 37°C. The restriction digest samples were analyzed
172 by electrophoresis in 5% nondenaturing polyacrylamide gels using a vertical gel electrophoresis
173 apparatus (BRL Model V16, Invitrogen, Carlsbad, CA). The DNA-banding profiles were
174 identified by ethidium bromide staining and were subsequently analyzed using the GelCompar II
175 software (Applied Maths, Inc., Austin, TX). For sequencing the 16S rDNA variable regions V1
176 and V2 (21), the primers (2) used for amplification of the 5' end (approximately 300 bases) of
177 the 16S gene were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-
178 GTCTCAGTCCCAATGTGGCC-3'. The cycle times and temperatures were: 10 min at 94°C; 25
179 cycles of 1 min at 94°C, 2 min at 61°C, and 2 min at 72°C; and 5 min at 72°C. Alternatively,

180 primers 5'-AGTTTGATCMTGGCTCAG-3' (M=A or C), and 5'-
181 AGGAGGTGATCCARCCGCA-3' (R=A or G) were used to amplify the entire 16S rDNA gene
182 (7) substituting an annealing temperature of 55°C. PCR products were purified using a
183 QIAquick® PCR purification kit (Qiagen Inc., Valencia, CA.) and the DNA fragments were
184 sequenced commercially (Davis Sequencing, Davis, CA). Sequences were analyzed by BLAST
185 (Basic Local Alignment Search Tool) search of the NCBI (National Center for Biotechnology
186 Information) non-redundant DNA sequence database (<http://www.ncbi.nlm.nih.gov/>).

188 RESULTS

189 **Fermentation microbiology and chemistry:** The shredded cabbage used for all
190 fermentations in this study contained between 4×10^6 and 6×10^6 cfu/g total aerobes, 2 to 3×10^6
191 cfu/g total *Enterobacteriaceae*, and less than 10^2 cfu/g yeast and mold. However, the initial LAB
192 populations from the shredded cabbage varied from 10^4 cfu/g in the Y1 cabbage to 10^6 cfu/g
193 from two of the Y2 cabbage samples. It is possible some growth of LAB occurred during
194 transport, prior to analysis. As previously reported (11, 22), during the first week of each
195 fermentation a rapid increase in total and lactic acid bacteria occurred, as well as a rapid decrease
196 in *Enterobacteriaceae* (data not shown).

197 Glucose and fructose were the primary fermentable sugars in the cabbage (between 1.5%
198 and 2.2%, respectively). Sucrose made up only a small amount of the fermentable sugars, less
199 than 0.2% of the cabbage by weight, and was not detectable in Y2 samples. Overall, the cabbage
200 used in Y1 contained more sugar than the Y2 samples. However, sugar utilization, acid
201 production, and pH profiles were similar in the commercial tanks from Y1 and Y2. The results
202 from chemical analysis indicated that the fermentation in the four commercial sauerkraut

203 fermentation tanks from Y1 (Fig. 1) and Y2 (not shown) were normal and consistent with those
204 described by Fleming et. al. (14) and Peterson and Albury (22). During the first week of
205 fermentation lactic acid, acetic acid and mannitol were produced. The pH on day 14 for all
206 fermentations was in the range of 3.4 to 3.7 (data not shown).

207 **ITS-PCR database and 16S rDNA sequence analysis:** A database of ITS-PCR gel
208 banding patterns was generated using 64 LAB strains from 42 different species (Suppl. Table 1
209 and Suppl. Fig. 1), using both the *RsaI* digested and undigested PCR products. The 686
210 fermentation isolates obtained from brine during the two year study were grouped by ITS-PCR
211 banding patterns using the GenCompar II software, for both the *RsaI* digested and undigested
212 PCR products. Banding patterns for the isolates were grouped by unique ITS-PCR banding
213 patterns, and 652 isolates tentatively identified using the ITS-PCR database. One or more
214 representative cultures from each group with unique banding patterns were then further
215 characterized by sequencing the 5' end of the 16S rDNA. The sequence accession numbers,
216 along with the corresponding bacterial strain identification by BLAST analysis, are shown in
217 Suppl. Data Table 2. By this method *L. mesenteroides* was identified as the predominant species
218 (179 of 686 isolates) in the early heterofermentative stage of fermentation and *L. plantarum* was
219 the predominant species involved in the late homofermentative stage (280 of 686 isolates).
220 Together, these two species made up 2/3 of all isolates (67%). *Weissella* sp. and *L. curvatus* were
221 the next most common species among the isolates, with 57 (8.3%) and 40 (5.8%) total isolates
222 from the two year study. The majority of LAB isolates (90% to 100%) taken from days 1 and 3
223 after the start of fermentation for the Y1 and Y2 samples were heterofermentative species.
224 Homofermentative LAB made up a similar majority of the isolates from day 14 (Figure 2A and
225 2B).

226 **Microbial diversity in commercial sauerkraut fermentations:** Three different
227 bacterial species were found in the Y1, day one samples: *L. mesenteroides*, *Weissella* sp., and *L.*
228 *citreum* (Table 2, Figure 3). As expected, *L. mesenteroides* was the predominant organism
229 associated with the fermentation, comprising 88% (84 out of 95) of the isolates from the first
230 day. *L. mesenteroides* was the most frequently isolated species in the brine samples from day 3,
231 but it contributed only 41% (38 out of 93) of total isolates. Other species found on day 3
232 included: *L. curvatus* (15%), *Weissella* sp. (14%), *L. fallax* (9%) and others (Table 2).
233 Homofermentative *L. plantarum* strains were observed in the fermentation samples from day 3,
234 but only accounted for 9.7% of the isolates. We observed variation in the pattern of hetero- and
235 homofermentative LAB between the Y1 and three Y2 fermentations. Samples from Tank 3 of Y2
236 were unique when compared to the samples from other tanks, because both heterofermentative
237 and homofermentative LAB were present in all samples.

238 As expected, bacterial isolates from Y1 days 7, 9 and 14 contained no heterofermentative
239 *L. mesenteroides* or *Weissella* sp. isolates. Overall, the diversity of species was greatest from the
240 day 9 samples from Y1, when 9 different species were isolated, out of a total of 90 isolates. The
241 majority of isolates from the brine samples taken during days 7, 9 and 14, were identified as *L.*
242 *plantarum* (68.8%, 56.7%, and 78.1%, respectively). *L. argentinum*, which has not previously
243 been found in sauerkraut fermentations, accounted for 10% of total isolates recovered from Y1
244 day 9. Interestingly, only one isolate of *P. pentosaceus* and 14 isolates of *L. brevis* were found
245 among the 467 total Y1 isolates. Of the 279 Y1 isolates from days 7, 9 and 14 a total of 28 were
246 not identified by ITS-PCR pattern or 16S rDNA sequencing.

247 Isolates from the three Y2 fermentation tanks had bacterial species profiles as described
248 in Table 3. In tank one, three heterofermentative LAB species were recovered from day one, *L.*

249 *mesenteroides*, *Weissella* sp., and *L. citreum*. However *Weissella* sp. represented the majority of
250 isolates recovered (12 of 20 in day 1, and 7 of 19 in day 3). *L. curvatus*, *L. fallax*, and *L.*
251 *plantarum* were also recovered from day 3 brine samples. *L. plantarum* was the major LAB
252 species isolated from the Y2 tank one sample from days 7 and 14, representing over 46% and
253 94% of isolates, respectively. The bacterial species from tank two profiles were similar to the
254 tank one fermentation (Table 3). At day 14, *L. plantarum* was the only LAB isolated from the
255 brine sample, accounted for 100% (18 of 18) of the isolates. In tank three, however, 8 different
256 species were identified from 17 isolates from day one. Only 1 *P. pentosaceus* and 1 *L. brevis*
257 isolate was recovered from the 219 total Y2 isolates.

258 **DISCUSSION**

259 We used the ITS-PCR database to identify major bacterial species present in commercial
260 sauerkraut fermentations. It is possible that different bacterial species could be present that have
261 very similar or indistinguishable ITS patterns, or that some bacterial species did not grow on
262 MRS medium. Because we only sequenced 16S DNA from isolates with representative ITS-PCR
263 patterns, we may have failed to identify some species that were present among the isolates. The
264 data presented, therefore, may underreport the microbial diversity in the sauerkraut
265 fermentations. Analysis of ITS-PCR patterns and 16S rDNA sequences from the isolates,
266 however, agreed with the biochemical analysis indicating that heterofermentative LAB species
267 dominated the first week of the commercial sauerkraut fermentations, and homofermentative
268 species were predominate in the second week. *Weissella* sp. were recovered together with *L.*
269 *mesenteroides* both in day one and three. We could not identify species of any *Weissella* sp.
270 isolates by 16S rDNA sequence comparisons with the GenBank database because of the
271

272 sequence similarity of *Weissella confusa*, *Weissella cibraria*, and *Weissella kimchii*. *Weissella*
273 species have previously been recovered from plant products (5, 8, 18), and have very similar 16S
274 rDNA sequences (3, 5). Complete sequencing of the 16S genes from selected *Weissella* isolates
275 did not allow conclusive identification with known species (data not shown). *Weissella* sp. were
276 previously characterized as members of the *Leuconostoc* genus (6). There was variation between
277 the three Y2 fermentations in microbiota from the heterolactic (days one and three) stage of
278 fermentation, which is believed to be vital to the quality of sauerkraut. This variation may be due
279 in part to the small sampling size (20 isolates from each sampling time, and from each
280 fermentation in Y2) but could represent differences in microbiota. These data indicate that the
281 use of *L. mesenteroides* starter cultures may help improve consistency in flavor development in
282 commercial sauerkraut.

283 During the transition period between the heterofermentation and homofermentation
284 phases of fermentation (days three to nine), a variety of LAB species including *L. curvatus* and
285 *Leuconostoc argentinum* were isolated. *L. argentinum* was originally isolated from raw milk in
286 Argentina (9). *L. plantarum* became the dominant microorganism after seven days into the
287 fermentation when the pH decreased to at least 3.9 or lower in all 4 fermentations. However, we
288 recovered relatively few *L. brevis* (15 total isolates of 686) and only two isolates of *P.*
289 *pentosaceus*. This was surprising because the previous research has shown that both were
290 considered to be major bacterial species involved in sauerkraut fermentation (22).

291 This study and a related study of the bacteriophage ecology of commercial sauerkraut
292 fermentations (15), has significantly revised the classical understanding (11, 22) of the
293 microbiota present in sauerkraut fermentations. Several LABs isolated from the two-year study
294 had never been previously recovered from sauerkraut fermentation including *Weissella* sp., *L.*

295 *argentinum*, *Lactobacillus coryniformis*, *Leuconostoc citreum*, *Lactobacillus paraplantaum*, and
296 *Lactobacillus paracasei*. This study, and a recent study of the comparative genomics of lactic
297 acid bacteria, including *L. mesenteroides* (16), may also aid in the development of starter
298 cultures, and increase our understanding of cabbage fermentation biochemistry and ecology.

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REFERENCES

- 307
- 308
- 309 1. Aguirre, M. and M. D. Collins. 1993. Lactic Acid Bacteria and Human Clinical Infection.
310 J. Appl. Bacteriol. **75**:95-107.
- 311 2. Barrangou, R., S.-S. Yoon, F. Breidt, H. P. Fleming, and T. R. Klaenhammer. 2002.
312 Characterization of Six *Leuconostoc fallax* Bacteriophages Isolated from an Industrial
313 Sauerkraut Fermentation. Appl. Environ. Microbiol. **68**:5452-5458.
- 314 3. Bjorkroth, K. J., U. Schillinger, R. Geisen, N. Weiss, B. Hoste, W. H. Holzapfel, H. J.
315 Korkeala, and P. Vandamme. 2002. Taxonomic study of *Weissella confusa* and description
316 of *Weissella cibaria* sp. Nov., detected in food and clinical samples. Int. J. Syst. Evol.
317 Microbiol. **52**:141-148.
- 318 4. Breidt, F. and H. P. Fleming. 1996. Identification of Lactic Acid Bacteria By Ribotyping.
319 Journal of Rapid Methods and Automation in Microbiology **4**:219-233.
- 320 5. Choi, H. K., C. C. Ick, K. S. Bo, L. J. Choul, L. D. Woo, C. S. Won, P. J. Min, and P. Y.
321 Ryang. 2002. *Weissella kimchii* sp. nov., a novel lactic acid bacterium from kimchi. Int. J.
322 Syst. Evol. Microbiol. **52**:507-511.
- 323 6. Collins, M. D., J. Samelis, J. Metaxopoulos, and S. Wallbanks. 1993. Taxonomics studies
324 on some *Leuconostoc*-like organisms from fermented sausages: description of a new genus
325 *Weissella* for the *Leuconostoc paramesenteroides* group of species. J. Appl. Bacteriol.
326 **75**:595-603.
- 327 7. Dasen, G., J. Smutney, M. Teuber, and L. Meile. 1998. Classification and identification of

- 328 Propionibacteria based on ribosomal RNA genes and PCR. *System Appl. Microbiol.*
329 **21**:251-259.
- 330 8. De-Vuyst, L., V. Schrijvers, S. Pararmithiotis, B. Hoste, M. Vancanneyt, J. Swings, G.
331 Kalantzopoulos, E. Tsakalidou, and W. Messens. 2002. The biodiversity of lactic acid
332 bacteria in Greek traditional wheat sourdoughs is reflected in both composition and
333 metabolite formation. *Appl. Environ. Microbiol.* **68**:6059-6069.
- 334 9. Dicks, L. M. T., L. Fantuzzi, F. C. Gonzalez, M. Du Toit, and F. Dellaglio. 1993.
335 *Leuconostoc argentinum* sp. nov., isolated from Argentine raw milk. *Int. J. Syst. Bacteriol.*
336 **43**:347-351.
- 337 10. Du Plessis, E. M. and L. M. T. Dicks. 1995. Evaluation of Random Amplified
338 Polymorphic DNA (RAPD)-PCR as a Method to Differentiate *Lactobacillus acidophilus*,
339 *Lactobacillus crispatus*, *Lactobacillus amylovorus*, *Lactobacillus gallinarum*, *Lactobacillus*
340 *gasseri*, and *Lactobacillus johnsonii*. *Curr. Microbiol.* **31**:114-118.
- 341 11. Fleming, H. P., Kyung, K. H., and Breidt, F. 1995. Vegetable Fermentations.
342 *Biotechnology*. 9, p. 629-661. 95. New York. Rehm, H.-J. and Reed, G.
- 343 12. Fleming, H. P., R. F. McFeeters, and M. A. Daeschel. 1985. The Lactobacilli, Pediococci,
344 and Leuconostocs: Vegetable Products. CRC Press, Inc., Boca Raton, FL.
- 345 13. Fleming, H. P., R. F. McFeeters, and M. A. Daeschel. 1992. Fermented and acidified
346 vegetables. In *compendium of methods for the microbiological examination of foods*, 3rd
347 edition (Vanderzant, C., and D. F. Splittstoesser, eds.), p. 929-952, American public health
348 association, Washington, DC.

- 349 14. Fleming, H. P., R. F. McFeeters, J. L. Etchells, and T. A. Bell. 1984. Pickled vegetables. In
350 *compendium of methods for the microbiological examination of foods*, 2nd edition (M. L.
351 Speck eds.), p. 663-681, American public health association, Washington, DC.
- 352 15. Lu, Z., F. Breidt, Jr., V. Plengvidhya, and H. P. Fleming. 2003. Bacteriophage ecology in
353 commercial sauerkraut fermentations. *Appl. Environ. Microbiol.* 69 (6):3192-3202.
- 354 16. Makarova, K., A. Slesarev, Y. Wolf, A. Sorokin, B. Mirkin, E. Koonin, A. Pavlov, N.
355 Pavlova, V. Karamychev, N. Polouchine, V. Shakhova, I. Grigoriev, Y. Lou, D. Rohksar,
356 S. Lucas, K. Huang, D. M. Goodstein, T. Hawkins, V. Plengvidhya, D. Welker, J. Hughes,
357 Y. Goh, A. Benson, K. Baldwin, J.-H. Lee, I. Díaz-Muñiz, B. Dosti, V. Smeianov, W.
358 Wechter, R. Barabote, G. Lorca, E. Altermann, R. Barrangou, B. Ganesan, Y. Xie, H.
359 Rawsthorne, D. Tamir, C. Parker, F. Breidt, Jr., J. Broadbent, R. Hutkins, D. O' Sullivan, J.
360 Steele, G. Unlu, M. Saier, T. Klaenhammer, P. Richardson, S. Kozyavkin, B. Weimer, D.
361 Mills. 2006. Comparative Genomics of the Lactic Acid Bacteria, 2006. Proceedings of the
362 National Academy of Sciences 103 (42):15611-15616.
- 363 17. McDonald, L. C., McFeeters, R. F., Daeschel, M. A., and Fleming, H. P. 1987. A
364 differential medium for the enumeration of homofermentative and heterofermentative lactic
365 acid bacteria. *Appl. Environ. Microbiol.* 53 (6):1382-1384.
- 366 18. Mugula, J. K., S. Nnko, J. A. Narvhus, and T. Sorhaug. 2003. Microbiological and
367 fermentation characteristics of togwa, a Tanzanian fermented food. *Int. J. Food Microbiol.*
368 **80**:187-199.
- 369 19. Murcia-Martinez, A. J. and M. D. Collins. 1990. A phylogenetic Analysis of the Genus

- 370 Leuconostoc Based on Reverse Transcriptase Sequencing of 16 S rRNA. FEMS Microbiol.
371 Lett. **70**:73-84.
- 372 20. Murcia-Martinez, A. J. and M. D. Collins. 1991. A phylogenic analysis of an atypical
373 leuconostoc: description of *Leuconostoc fallax*. FEMS Microbiol. Lett. **82**:55-60.
- 374 21. Neefs, J. M., Y. Van de Peer, P. De Rijk, S. Chapelle, and R. De Wachter. 1993.
375 Compilation of small ribosomal subunit RNA structures. Nucleic Acids Res. **21**:3025-
376 3049.
- 377 22. Pederson, C. S. and Albury, M. N. The Sauerkraut Fermentation. 1969. N.Y. State Agr.
378 Expt. Sta. (Geneva, N.Y.) Tech. Bull. Bulletin 824.
- 379 23. Plengvidhya, V., F. Breidt, and H. P. Fleming. 2004. Use of RADP-PCR as a method to
380 follow the progress of starter cultures in sauerkraut fermentations. Int. J. Food Microbiol.
381 **93**:287-296.
- 382 24. Schleifer, K. H., M. Ehrmann, C. Beimfohr, E. Brockmann, W. Ludwig, and R. Amann.
383 1995. Application of Molecular Methods for the Classification and Identification of Lactic
384 Acid Bacteria. International Dairy Journal **5**:1081-1094.
- 385 25. Vogel, R. F., M. Lohmann, A. N. Weller, M. Hugas, and W. P. Hammer. 1991. Structural
386 similarity and distribution of small cryptic plasmids of *Lactobacillus curvatus* and *L. sake*.
387 FEMS Microbiol. Lett. **84**:183-190.
- 388 26. Ward, D. M., R. Weller, and M. M. Bateson. 1990. 16S rRNA Sequences Reveal
389 Numerous Uncultured Microorganisms in a Natural Community. Nature **345**:63-65.

FIGURE LEGENDS

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392 Figure 1. Biochemistry of the Y1 fermentation. Changes in the concentrations of acids and
393 sugars during the first 14 days after the start of the Y1 fermentation are shown: lactic acid
394 (circles); acetic acid (diamonds); mannitol (triangle up); glucose (squares); fructose (triangle
395 down).

396

397 Figure 2. Changes in gas production microorganisms during commercial sauerkraut fermentations.
398 The percent of total isolates that were heterofermentative (A) or homofermentative (B) at each
399 day (1, 3, 7, and 14) for each of the four commercial fermentations sampled (represented by the
400 shaded bars) are shown.

401

402 Figure 3. The microbial diversity of the Y1 fermentation as determined by ITS-PCR and 16S
403 rDNA sequencing. For the total number of isolates on days 1, 3, 7, and 14, (95, 93, 93, and 90,
404 respectively), the percent represented by each species is indicated by the bars.

405

406 Figure 4. The microbial diversity of the Y2 fermentation as determined by ITS-PCR and 16S
407 rDNA sequencing. For the total number of isolates on days 1, 3, 7, and 14, (95, 93, 93, and 90,
408 respectively), the percent represented by each species is indicated by the bars. Results from tank
409 1 (A), tank two (B), and tank three (C) are shown.

410 **Table 1.** Biochemistry of cabbage used in sauerkraut fermentations

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Year/fermentation	Glucose		Fructose		Sucrose		Malic acid	
	mM	%	mM	%	mM	%	mM	%
1/1	119.2	2.15	90.8	1.64	4.8	0.17	5.6	0.08
2/1	92.4	1.66	81.7	1.47	ND ^b	ND	3.9	0.05
2/2	90.8	1.63	83.5	1.50	ND	ND	3.5	0.05
2/3	81.7	1.47	73.8	1.33	ND	ND	4.7	0.06

425 ^amM = millimolar, percentage by weight.

426 ^bND = not detected.

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Table 2: Identification of Y1 Isolates

Species	Day 1	Day 3	Day 7	Day 9	Day 14
<i>L. mesenteroides</i>	84	38			
<i>Weissella</i> sp.	7	20			
<i>L. citrium</i>	4	1			
<i>L. curvatus</i>		14	14	3	
<i>L. fallax</i>		8	1	1	
<i>L. plantarum</i>		9	64	51	75
<i>L. brevis</i>		3	3	3	5
<i>L. coryniformis</i>			5	1	
<i>L. argentinum</i>			1	11	
<i>P. pentosaceus</i>			1		
<i>L. paraplantarum</i>			3	6	3
NID*			1	14	13
Samples analyzed**	95	93	93	90	96

* NID = Not identified

**Total number of samples analyzed

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Table 3. Identification of Y2 isolates

Tank 1				
Species	Day 1	Day 3	Day 7	Day 14
<i>L. mesenteroides</i>	6	2	2	
<i>Weissella</i> sp.	12	7		
<i>L. citrium</i>	2			
<i>L. curvatus</i>		5	3	
<i>L. fallax</i>		1	1	
<i>L. plantarum</i>		3	7	16
<i>P. pentosaceus</i>		1		
<i>L. paraplantarum</i>			1	1
ND*			1	
Samples analyzed**	20	19	15	17
Tank 2				
Species	Day 1	Day 3	Day 7	Day 14
<i>L. mesenteroides</i>	12	16	7	
<i>Weissella</i> sp.	2			
<i>L. citrium</i>			1	
<i>L. fallax</i>		2	1	
<i>L. plantarum</i>	1		9	18
<i>L. lactis</i>		1		
<i>L. argentinum</i>	4			
<i>L. mali</i>			1	
Samples analyzed	19	19	19	18
Tank 3				
Species	Day 1	Day 3	Day 7	Day 14
<i>L. mesenteroides</i>	1	9	2	
<i>Weissella</i> sp.	1	7	1	
<i>L. citrium</i>	4	1		
<i>L. curvatus</i>			1	
<i>L. fallax</i>	2	2	4	
<i>L. plantarum</i>	1		10	16
<i>L. brevis</i>				1
<i>L. argentinum</i>	1			
<i>L. paracasei</i>	4			
NID	3	1		1
Samples analyzed	17	20	18	18

* NID = Not identified

** Total number of samples analyzed

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Figures for Eddie paper 1

ACCEPTED

Figure 1

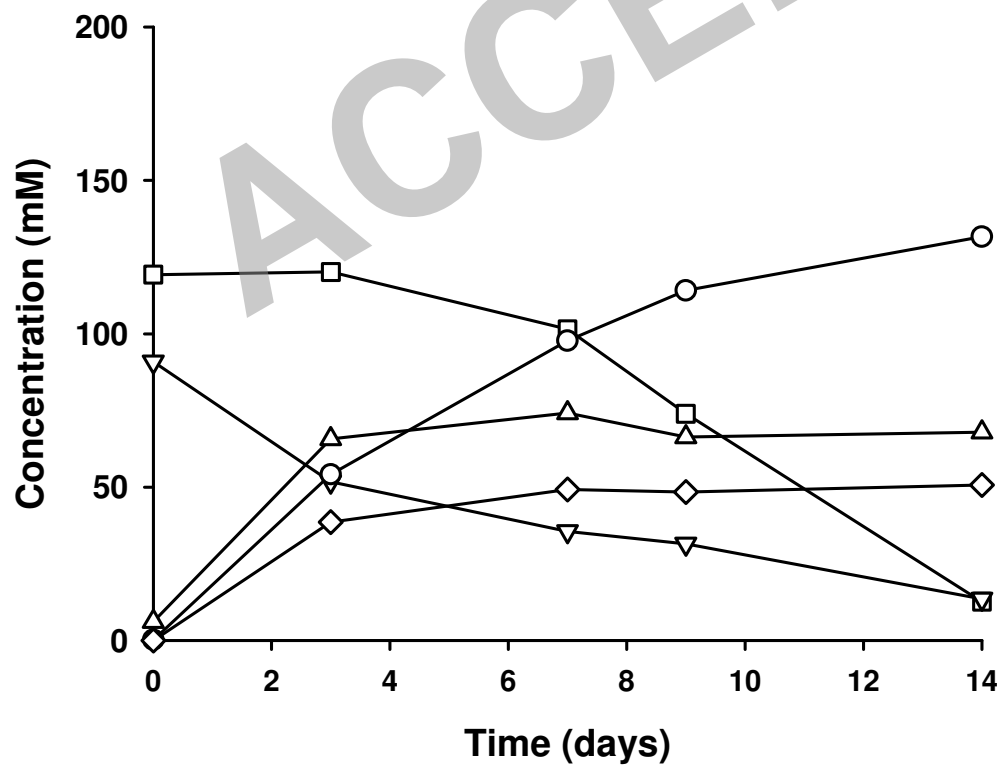


Figure 2

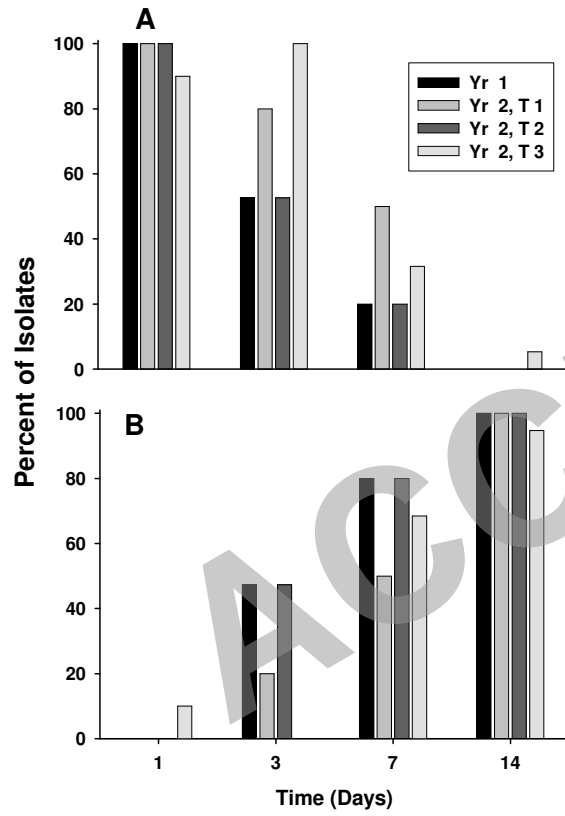


Figure 3

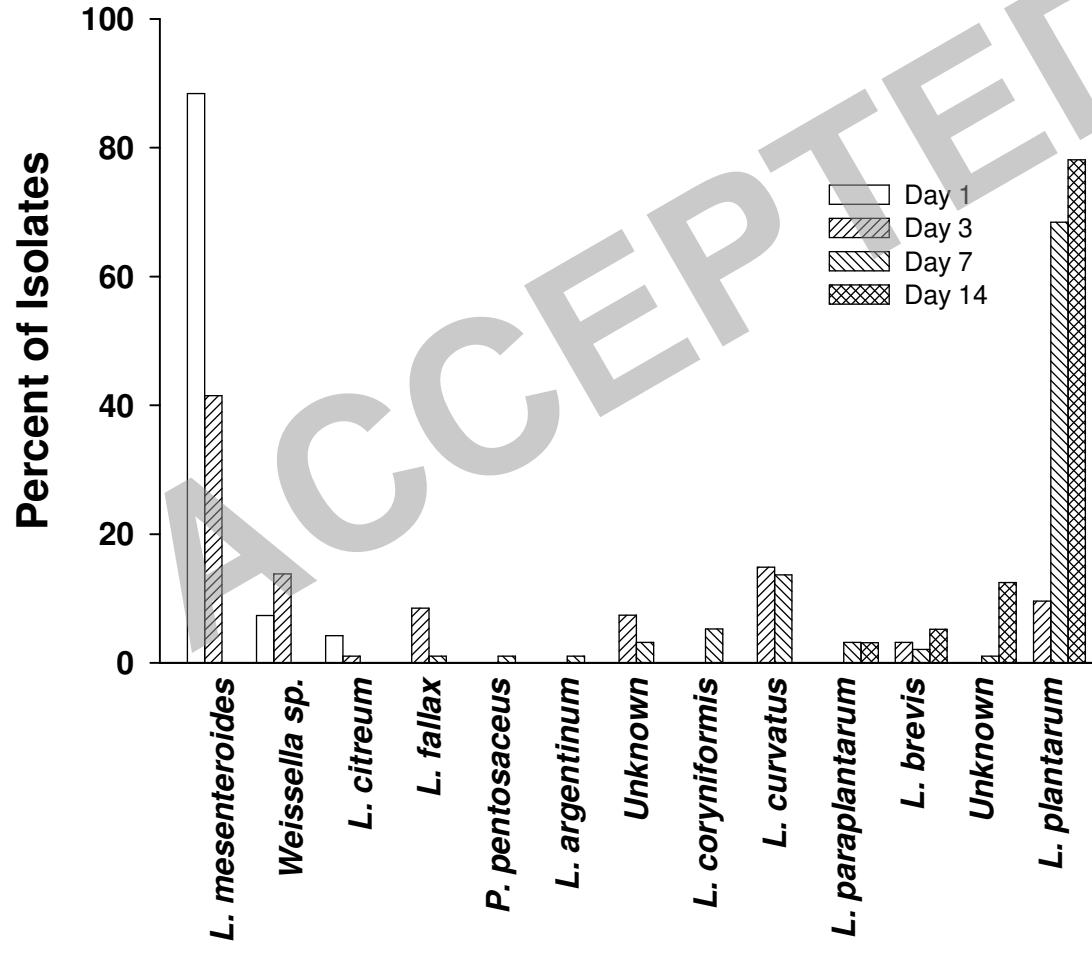


Figure 4

