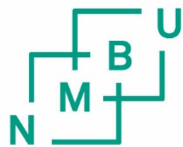


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# A Comparative Analysis on Phylogeny, Genetics and Selected Phenotypes of Lactic Acid Bacteria Isolated from Gut Microbiota of Honey Bee Versus Flowers

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The experimental part of this work was performed at the Laboratory of Microbial Gene Technology (LMG).

## Abstract

From the bee gut samples 190 bacteria isolates were obtained. Purified plasmid DNA extracts were screened for plasmids on an agarose gel by electrophoresis to determine their plasmid profiles. The purpose of the plasmid DNA profiling was to identify different strains of the same species later on in the experiment. Based on the plasmid profiles, 99 isolates were selected for 16S rDNA sequencing following a phylogenetic analysis of the 16S rDNA sequence. Further phenotypic characteristics were tested by determining the carbohydrate metabolism of 20 strains belonging to each genus or species of genera identified by the analysis of the 16S rDNA. Finally, all strains were screened for antimicrobial activity, bacteriocins. In a similar experiment conducted parallel to the gut microbiota, five samples from bacterial flora of flowers, sampled nearby the bee colonies, were conducted by another student.

The results show a significant amount of lactic acid bacterial species known to be inhabitants of the bee gut microbiota, as well as *Bifidobacteria.* The other species represented in the samples, identified 16S rDNA, are also consider as bacteria common to the bees. The comparison of identified lactic acid bacteria of isolated strains from bees and flower had various amounts of agreement looking at all isolates from all four months of sampling, however, in the early the summer months the microbiota was more similar. The study of phylogeny, genetics and phenotypical characteristics conducted in this thesis have been comprehensive. More work on bacteriocin screening, such as testing other methods, would be interesting in future work. Future work on the phylogenetic placement of Lactobacillus kunkeei group 2, are especially interesting since it does not branch closely with any of the type strains in phylogenetic trees produced.

## Sammendrag

Fra de fem bie-tram mikrobiota prøvene ble 190 bakteriestammer isolert. Renset plasmid DNA ble screenet for plasmider ved hjelp av agarose-gelelektroforese for å bestemme bakterie isolatenes plasmidprofil. Formålet med plasmid DNA profileringen var å kunne differensiere identifiserte bakteriestammer av samme art senere i forsøket. Basert på plasmidprofilene, ble 99 isolater valgt ut for 16S rDNA-sekvensering etterfulgt av en fylogenetisk analyse av 16S rDNA-sekvensene. Ytterligere fenotypiske egenskaper ble testet hos 20 stammer ved bestemmelse av karbohydratmetabolismen/fermenteringsprofilen til de ulike artene identifisert i 16S rDNA analysen. Til slutt ble alle stammer testet for antimikrobiell aktivitet, bakteriosiner. Parallelt med bie-tarm mikrobiota undersøkelsene ble 5 prøver fra blomster undersøkt med tilsvarende metode.

Resultatene viser at det er en betydelig mengde melkesyrebakterier som er kjent for å være beboere av denne fruktofile nisjen som finnes bie tarm mikrobiotaen, i tillegg til bifidobakterier. De andre artene som er representert i prøvene, identifisert ved 16S rDNA er også betrakte som bakterier som er felles for biene. Sammenligningen av identifiserte melkesyrebakterier av isolerte stammer fra bier og blomster hadde disse ganske ulik sammensetning om man ser på identifiserte stammer fra alle fire måneder med prøvetaking, men i begynnelsen av sommermånedene derimot, var mikrobiotaen mer lik. Studiet av fylogeni, genetikk og fenotypiske egenskaper utført i denne avhandlingen har vært omfattende. Mer arbeid på bakteriocin screening, for eksempel ved å benytte andre metoder, ville være interessant i et videre arbeid. Fremtidig arbeid med den fylogenetiske plasseringen av Lactobacillus kunkeei gruppe 2, er spesielt interessant siden fylogenetisk plassering av typestammer i de fylogenetiske trærne som har blitt laget hadde stor avstand.

59 # Abbreviations

| | | |
|---|---|---|
| 60 | BLAST | Basic Local Alignment Tool |
| 61 | bp | base pair(s) |
| 62 | CCD | Colony Collapse Disorder |
| 63 | CFU | Colony forming units |
| 64 | $dH_2O$ | Distilled water |
| 65 | FLAB | Fructophilic Lactic Acid Bacteria |
| 66 | g | gram(s) |
| 67 | GRAS | Generally regarded as safe |
| 68 | LAB | Lactic Acid Bacteria |
| 69 | min | Minute(s) |
| 70 | NCBI | National Center for Biotechnology Information |
| 71 | NMBU | Norges Miljø- og Biovitenskapelige Universitet |
| 72 | ON | Over Night |
| 73 | PBS | Phosphate buffered saline |
| 74 | PCR | Polymerase chain reaction |
| 75 | PTM | Post translational modifications |
| 76 | rDNA | Ribosomal Deoxyribonucleic acid |
| 77 | RDP | Ribosomal Database Project |
| 78 | rRNA | Ribosomal ribonucleic acid |
| 79 | rpm | Revolutions per minute |
| 80 | RT | Room Temperature |
| 81 | s/sec | Second(s) |

82

83

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138 # 1. Introduction

139 ## 1.1 Lactic Acid Bacteria

140 Lactic acid bacteria are Gram-stain-positive bacteria in phylum *Firmicutes*. They are found
141 The *Lactobacillus* bacteria are facultative anaerobes, non-motile, non-spore forming, and rod-
142 shaped bacteria that are found in a variety of environments, such as dairy products, fermented
143 foods, and as normal inhabitants in the gastrointestinal tract of both humans and animals
144 (Jeyaprakash et al., 2003, Endo et al., 2015). As inhabitants of the gastrointestinal tract, they
145 are known to be involved in immunomodulation, interference with pathogenic enteric
146 bacteria, and upholds a healthy intestinal microflora (Jeyaprakash et al., 2003).
147 Bifidobacterium are anaerobic
148 Two *Lactobacillus* spp., have been classified as fructophilic, namely *L. kunkeei* and *L. florum*
149 (Neveling et al., 2012). *Lactobacillus kunkeei* is an obligatory heterofermentative fructophilic
150 lactic acid bacteria (FLAB), growth on D-glucose in the presence of external electron
151 acceptors such as pyruvate, oxygen and fructose, *L. kunkeei* has enhanced growth as opposed
152 to poor growth on glucose under anaerobic conditions. *L. florum*, on the other hand, ferments
153 both fructose and glucose anaerobically, although the former being fermented more
154 efficiently. *L. florum* is therefore classified as a facultative fructophilic LAB. The other
155 obligately (heterofermentative) fructophilic LAB obligately fructophilic are grouped in the
156 genus *Fructobacillus*, which is closely related to to genera *Leuconostoc*, *Oenococcus* and
157 *Weissella* (Neveling et al., 2012, Endo et al., 2015). The genus *Fructobacillus* in the family
158 *Leuconostocaceae* is comprised of five species: *F. fructosus* (type species), *F. durionis*, *F.*
159 *ficulneus*, *F. pseudoficulneus* and *F. tropaeoli*, where four of the five species were until
160 recently considered as members of the *Leuconostoc* genus. The reclassification was based on
161 their phylogenetic position, morphology and biochemical characteristics (Endo et al., 2015).
162 Endo et al. (2015) found that on glucose only medium the growth of *Fructobacillus* was poor
163 under anaerobic conditions and good growth under aerobic conditions, while *Leuconostoc*
164 grew well in both anaerobic and aerobic conditions and the effect of oxygen on *Leuconostoc*
165 was generation of smaller colonies.
166 *Lactococcus lactis* is a LAB that is extensively used in the food industry for its fermenting
167 properties, such as in starter cultures for the production of cheese and kefir (fermented milk).
168 Lactococcus lactis strains are known to carry plasmids encoding lactose catabolism,
169 proteinase production, citrate utilisation, antibiotic and heavy metal resistance, and

170 bacteriocin production and immunity, all are industrially important traits that have been
171 extensively studied (Siezen et al., 2005).

## 172 1.2 Gut Microbiota of Honey Bees

### 173 1.2.1 Transmission of Microbes Between Honey Bees and Their Offspring

174 The midgut of the insect is the primary site of nutrient uptake from enzymatic digestion.
175 Honey bees (*Apis mellifera*) are important in food production and the world agriculture for their
176 prominent pollination of crops. They provide honey, wax, and other products that human take
177 advantage of in for example food (Evans and Schwarz, 2011). They shape ecosystems, together
178 with other insects, by contributing to the gene flow of angiosperm between flowers during
179 foraging for food. In return, flowers provide bees pollen and nectar, which is the main source
180 of nutrition, having all the vitamins, proteins and sugars they need for colony expansion. The
181 nutritional important benefits of bee pollen are the high concentrations of reducing sugars,
182 amino acids, fatty acids, minerals and significant quantities of vitamins as opposed to sugar
183 replacements given to bees in the winter season in Norway, however, this depends on the source
184 of pollen (Campos et al., 2010, Ludvigsen et al., 2015, Evans and Schwarz, 2011).
185 Honey bee embryos harbour few microbes (Evans and Schwarz, 2011). The origin of viral
186 infections in honey bee offspring is unclear. It could have been transmitted vertically or
187 horizontally to the offspring. Viral genomes have been found in semen of drones, which could
188 be a root of vertical transmission, but the honey bee queens could also transmit infections to
189 their offspring horizontally. As the queen produce tens of thousands of offspring in her life
190 time, a vertical or horizontal transmission of undesirable microbes is a considerable risk in
191 honey bee management where bees are moved from orchards to orchards for their commercial
192 application in pollination (Evans and Schwarz, 2011).
193 In the United States several varieties of orchards, such as almonds, apples, blueberries,
194 strawberries and canola are dependent on the honey bees for pollination and to accomplish
195 valuable yields. The economic value of commercial honeybee pollination is estimated at over
196 US $14 billion and $220 billion in the USA and worldwide, respectively. The consequence of
197 migratory beekeeping (moving the bees from orchards to orchards) could be the reason for the
198 massive mortality of domesticated honey bees due to colony collapse disorder (CCD), which
199 in turn also could lead to crop failure (Rangberg et al., 2012, Carroll et al., 2010, Vasquez et
200 al., 2012). Research conducted to understand the microbial interaction, both by the pathogenic
201 and symbiotic microbes, and to prevent the disorder, have found indications that the honey bees
202 carry novel microbiota dissimilar to other animals (Endo and Salminen, 2013).

203 ### 1.2.2 Bee gut bacteria of Apis mellifera worker bees and larvae

204 The microbiota of honey bees has been reported to be composed of several novel species of
205 lactobacilli. The bacterial community that seems consistent in honey bees belongs to several
206 proteobacteria (α, β, and γ), *Firmicutes*, *Tenericutes*, *Bacteroidetes*, and *Actinobacteria*.
207 Lactic acid bacteria (LAB) and fructophilic LAB, Lactobacillus, Lactococcus, Leuconostoc
208 and Bifidobacterium. The most abundant operational taxonomic units (OTU) belong to
209 Proteobacteria and *Firmicutes* in the honey bee guts, where the *Lactobacillales* dominate the
210 adult honey bee gut (Ahn et al., 2012). The honey bee larvae have few bacteria compared to
211 adult bees, but the microbiota composition are taxonomically similar (Anderson et al., 2016)
212

213 ## 1.3 16S rDNA

214 The molecular basis for identification of bacterial species are amplification and sequencing of
215 the 16S rDNA, followed by comparison of the obtained sequence with sequences in an
216 existing database. The 16S rDNA encodes the 16S rRNA gene involved in the small subunit
217 of the bacterial ribosome. The advantage of comparing 16S rRNA gene sequences for
218 identification are the extremely large amount of 16S rDNA sequences in databases. More than
219 60,000 16S rRNA gene sequences are available in the ribosomal Database Project (RFP II).
220 The 16S ribosomal RNA is a component of the 30S small ribosomal subunit. The prokaryotic
221 ribosome is about 1,500 kb long. The 16S rRNA gene performs the same function in all
222 organisms, and the gene is sufficiently conserved, containing conserved and variable regions.
223 The conserved regions are functionally constant, and gives important information about the
224 higher phylogenetic taxa, while the variable regions are evolutionary closer in time and are
225 often variable enough to clearly differentiate between species, and even strains of species
226 (Das and Dash, 2015, Rudi et al., 2007)
227

228 ## 1.4 Bacteriocin

229 It is believed that all organisms produce ribosomally synthesised antimicrobial peptides, and
230 in bacteria these are called bacteriocins. These antimicrobial peptides are an important part of
231 a bacteria's defence system. Bacteriocins are ribosomally synthesised and those that contain
232 post-translationally modifications consisting of the rare amino acid lanthionine are called
233 lantibiotics. Lantibiotics are made by modifications to various amino acids, such as serine-
234 and threonine residues that are dehydrated and then the thiol group of a cysteine is added.
235 This gives the characteristic lanthionine ring structure of lantibiotic peptides. genes are a

236 common feature among Gram-positive bacteria. Encoded by the genes of the respective
237 bacteria, whereas antibiotics are from, or chemically derived, naturally occurring enzyme
238 complexes (Villa and Vinas, 2016, Nes et al., 2007)

## 239 2. Materials and Methods

240 In January and February 2016, samples from the gut microbiota of honey bees, collected
241 during the summer of 2015 from an apiary in Ås, was used as sample material in this work.
242 The bee gut samples were chosen based on the date closest to bacterial samples collected
243 from different flowers (Linjordet, 2016). Selection of flowers were decided after consultation
244 with beekeeper Claus D. Kreibich, based on which flowers the bees were foraging at a given
245 time point. The samples from bees and flowers have been compared and analysed in different
246 ways, to see if there were any correlation between same and later time points with respect to
247 bacterial composition, and similarities and variances in phenotype and genotype of the genera
248 or species found within and between the two sources.
249 The bee gut samples collected by other students, that have been used in this work had been
250 prepared for long time storage in cryotubes at -80˚C with 750 µL 20% glycerol in PBS-buffer.
251

252 Table 1. The samples called C3T6, C3T7, C3T9, C3T12 and C3T13, was collected on May
253 13th and 27th, June 24th, and August 6th and 19th respectively.

| BEE SAMPLE | TIME POINT |
|---|---|
| C3T6 | May 13th |
| C3T7 | May 27th |
| C3T9 | June 24th |
| C3T12 | August 6th |
| C3T13 | August 19th |

254

255 Table 2. Sampling dates of flower samples that were chosen based on the diet of honey bees
256 during the foraging season

| FLOWER SAMPLE | TIME POINT |
|---|---|
| Dandelion | May 15th |
| Canola & apple flower | May 27th |
| Raspberries | June 22nd |
| Willow herb | August 19th |
| Willow herb | August 19th |

257

258 ## 2.1 Materials

259 ### 2.1.1 Bacterial strains

260 Table 3. Bacteria that has been used in this study. The isolated strains are not in this table.

| NAME | LMGT NUMBER | GROWTH TEMPERATURE (˚C) | COMMENTS |
|---|---|---|---|
| *Lactobacillus plantarum* 965 | B1525 | 30 | Used as indicator strain in bacteriocin screening |
| *L. sakei* 2313 | B487 | 30 | Used as indicator strain in bacteriocin screening |
| *Listeria innocua* | B486 | 30 | Used as indicator strain in bacteriocin screening |
| *Lactococcus lactis* IL403 | B1 | 30 | Used as indicator strain in bacteriocin screening |
| *L. garvieae* | 1546 | 30 | Broad spectrum producer of the bacteriocin GarKS (Garvicine KS), used as control bacteriocin screening |

261 ### 2.1.2 Equipment and instruments

262 Table 4. the equipment and instruments that has been used in this study. The table shows type of instrument or
263 equipment, which model the instrument was and the manufacturer.

| TYPE | MODEL | MANUFACTURER |
|---|---|---|
| Balance | XF-3200 | Salter |
| Centrifuge, large | Centrifuge 5804 R | Eppendorf, Germany |
| Centrifuge, small table-top | Microfuge® 16 Centrifuge | Beckman Coulter |
| Electrophoresis apparatus | Mini-Sub Cell®GT | Bio Rad® |
| Gas burner | Fireboy | Integra Biosiences |
| Ice Machine | KF85 | Porkka |
| Laminar Flow Cabinet | TL 2472 | HOLTEN |
| Nanodrop spectrophotometer | | NanoDrop® |
| Power Supply | PS 500XT | Hoefer Scientific Instruments |
| UV Molecular Imager | The Molecular Imager® Gel Doc™ XR Imaging System with Quantity One 1-D analysis software (v.4.6.7) | Bio-Rad laboratories, USA |
| Vortex mixer | Lab Dancer Mini | VWR |

| | Cat. No. 444-0004 | |
|---|---|---|
| Vortex mixer | MS2 Minishaker | IKA® |
| Water bath | U3 | Julabo |

264 ### 2.1.3 Kits

| KIT NAME | SUPPLIER |
|---|---|
| AnaeroGen™ 3.5L | Thermo Scientific |
| E.Z.N.A.® Plasmid DNA Mini Kit II Spin Protocol | Omega bio-tek |
| NucleoSpin® Gel and PCR Clean-up | Macherey-Nagel |
| API 50 CH | bioMérieux® sa, France |
| API 50 CHL medium for API 50 CH system | bioMérieux® sa, France |

265 ### 2.1.4 Chemicals

| NAME OF CHEMICAL | SUPPLIER |
|---|---|
| Acetic Acid (glacial) | Merck |
| Isopropanol | Arcus |
| MRS (de man, Rogosa, Sharpe) broth | Oxoid |
| Mineral Oil | Sigma |
| peqGREEN | VWR peqLab |
| UltraPure™ Agarose | Thermo Fisher Scientific |
| Tritiplex III (EDTA) | Merck |
| Trizma® Base (Tris-Base) | Sigma® |
| Trizma® hydrochloride (Tris-HCl) | Sigma® |
| OneTaq® DNA polymerase (#M0480L) | New England BioLabs® |
| OneTaq® Standard Reaction Buffer (5X) (#B9022S) | New England BioLabs® |

266 ### 2.1.5 Enzymes

| ENZYME | ACTIVITY | SUPPLIER |
|---|---|---|
| Lysozyme | Catalyse cleavage of peptidoglycan in the cell wall of Gram positive bacteria. | Sigma-Aldrich |
| Mutanolysin | Catalyse cleavage of peptidoglycan in the cell wall of Gram positive bacteria. | Sigma-Aldrich |
| RNase A | Degrades RNA | Omega bio-tek |

| | | Constituent part of the E.Z.N.A.® Plasmid DNA Mini Kit II Spin Protocol |
|---|---|---|
| SpeI (#R0133S) with CutSmart Buffer | Cuts DNA sequence at A/CTAGT | New England BioLabs® Inc. (NEB) |
| XbaI (#R0146S) with CutSmart Buffer | Cuts DNA sequence at T/CTAGA | New England BioLabs® Inc. (NEB) |
| XhoI with Buffer 4 | Cuts DNA sequence at C/TCGAG | New England BioLabs® Inc. (NEB) |

267

268 ### 2.1.6 Primers for PCR and sequencing of 16S rDNA

| PRIMER | | SEQUENCE (5´→3´) |
|---|---|---|
| 11F (forward primer) | 50-70 | 5´-TAA CAC ATG CAA GTC GAA CG-3´ |
| 4R (reverse primer) | 1392-1406 | 5´-ACG GGC GGT GTG TRC-3´ |

269

270 ### 2.1.7 Buffers

271 **TAE buffer** (Tris Acetic EDTA buffer):

272 **50x TAE** stock solution: 700 mL $dH_2O$ was added 242 g Tris-Base, 57.1 mL glacial
273 acetic acid (anhydrous acetic acid), and 100 mL 0.5M EDTA, the volume was adjusted to 1L
274 with more $dH_2O$ and stirred.

275 **1x TAE** buffer, working solution: 100 mL of 50x TAE buffer was added to 5 litres of
276 $dH_2O$ and stirred.

277 **TE-buffer** (Tris EDTA): 1mM Tris-HCl pH 8.0 and 100µM EDTA pH 8.0.

278 ### 2.1.8 Growth media

279 **MRS medium** (de man, Rogosa, Sharpe): 52 g of MRS broth (Oxoid) were completely
280 dissolved in 1 L of $dH_2O$ by adding a magnet into the flask and the flask is then placed on a
281 magnet stirrer.

282 **MRS-agar:** 3.75 g Agar-agar (Merck) is added to 250 mL MRS media in 500 mL flasks. The
283 MRS-agar was then autoclaved.

284 **MRS with fructose (40%) culture media**: Prepare MRS medium as described above. 7.5
285 mL sterile fructose 40% was added to 500 mL MRS medium in the sterile hood, stirred with
286 magnet stirrer (a small magnet has been dropped in the flasks when the MRS was made).

287 Distribute 6 mL of the MRS + fructose 40% into culture tubes firmly closed with culture-tube
288 hoods, and then autoclaved.
289 **MRS-soft agar**: MRS media was prepared as described above, volume adjusted to 100 mL.
290 5.2 g MRS-broth (Oxoid) was added 100 mL of $dH_2O$ and 0.8 g of Agar-agar (Merck), and
291 then autoclaved in bottles of 250 mL.
292 **NEBuffer 4 (10x)** (New England BioLabs)**:** 20 mM Tris-acetate, 10mM magnesium acetate,
293 50 mM potassium acetate, 1mM dithiothreitol (pH 7.9 at 25˚C)
294 **NEBuffer CutSmart® (10x)** (New England BioLabs): 20 mM Tris-acetate, 10 mM
295 magnesium acetate, 50 mM potassium acetate, 100 µg/ml BSA (pH 7.9 at 25˚C).

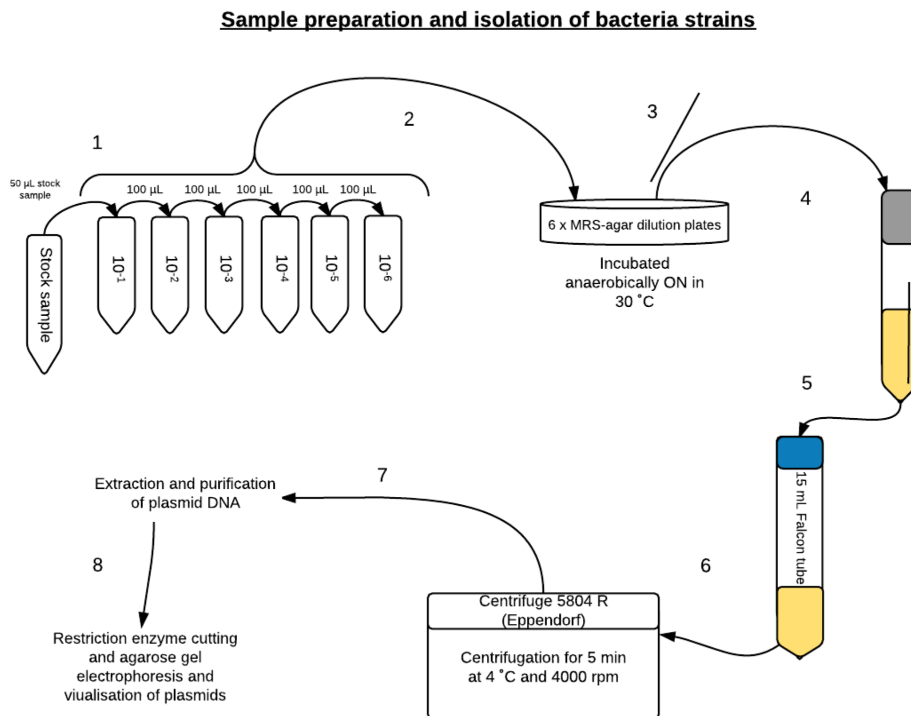
## 296 2.2 Methods

297 The results are divided into sub-chapters. The first sub-chapter is the pilot study, where some
298 of the bacterial isolates are analysed to determine how well the chosen solutions, procedures
299 and the experimental setups are performing for the isolation of our bacteria. Here we ended up
300 with some optimisations of the experimental setup in the growth experiments, which enzymes
301 and chemicals that were to be used, solution volumes, and determined how many dilution
302 series the stock samples needed.

### 303 2.2.1 The Pilot Experiment – Work Technique and Optimisation

304 The pilot experiment was done over a period of 8 days on a limited set of bacterial strains. Six
305 colonies from a -1 diluted sample was picked and inoculated on 6 mL MRS + fructose that was
306 incubated ON in 30 ˚C. This part of the pilot experiment is illustrated in Figure 1.The ON MRS
307 + fructose cultures were centrifuged and the pellets was collected and purified with the
308 E.Z.N.A.® plasmid DNA Mini kit II (Omega bio-tek). The amount of Elution Buffer used in
309 the pilot was 50 µL. The samples were eluted two times into two separate eppendorf tubes, for
310 the purpose to check if a second elution step was necessary or not. The rest of the protocol was
311 performed as described later. Both cut and uncut plasmid DNA product was run on an agarose
312 gel electrophoresis. The restriction enzymes SpeI (New England BioLabs) and XhoI (New
313 England BioLabs) were used to cut the purified plasmid DNA. The restriction enzymes were
314 tested individually and in combination. Decisions made based on these results were; the plasmid
315 product had to be eluted two times, since the second eluate had a large amount of DNA, and
316 the amount of elution buffer was reduced to 30 µL. A combination of the restriction enzymes
317 were shown favourable, however, the restriction enzyme XhoI (New England BioLabs) was
318 replaced by XbaI (New England BioLabs) assuming that the low quantity of observed plasmid
319 bands was due to XhoI (New England BioLabs) recognising sites that would require DNA

320 sequences with higher G+C content than SpeI (New England BioLabs). XbaI (New England
321 BioLabs), which is more suitable for low G+C genomes such as the lactobacilli, was therefore
322 chosen.
323




324
325 Figure 1. Flow diagram of the preparations and the isolation of the bacterial strains.

326 1) Each tube was added 900 µL 0.9 % NaCl, and then 50 µL sample. 2) 100 µL of each dilution
327 were transferred to MRS-agar plates and incubated anaerobically ON at 30 ˚C. 3) Six isolates
328 from the dilution plate with an appropriate amount of colonies were picked up with toothpicks.
329 4) The colonies were inoculated in 6 mL MRS + fructose culture tubes and incubated ON at 30
330 ˚C. 5) The cultures were transferred to falcon tubes and 6) centrifuged for 5 min at 4˚C and
331 4000 rpm. 7) The culture media was discarded and the pellets were used in the plasmid DNA
332 extraction. 8) Purified plasmid DNA was cut with restriction enzymes and visualised under UV
333 light.

334 **2.2.2 Isolation, Cultivation and Storage of Bacterial Strains**

335 All bacterial strains were grown on MRS-agar anaerobically at 30 ˚C. All the cultivated
336 bacterial strains were grown in MRS medium (MRS broth, Oxoid) with 15 % (v/v) fructose at
337 30 ˚C without shaking. Stock solutions were stored in -80 ˚C. PCR products and other genetic
338 materials were stored in -20 ˚C.

339 In the bacteriocin screening, all bacterial strains were initially grown on MRS-agar, while some
340 strains were later grown in BHI medium anaerobically and MRS medium ON at 30 ˚C.

### 341 2.2.3 Isolation of Bacteria and Purification of Plasmid DNA

342 From each bee gut microbiota sample:
343 1) 50 µL were taken out and diluted 4 times in 900 µL 0.9 % NaCl
344 2) 100 µL of each dilution was distributed onto MRS-agar plates and incubated
345 anaerobically ON at 30 ˚C
346 3) 40 colonies were pure cultured by picking up one colony and streaking it out on a new
347 MRS-agar plate
348 4) One colony from each pure culture was inoculated and grown in 6 mL MRS-broth +
349 fructose (40 %), at 30 ˚C ON.
350 5) Stock solutions were prepared by taking 800 µL of the culture and mixing it with 400
351 µL glycerol (45 %) to a final of concentration of 15 % (v/v) glycerol.
352 E.Z.N.A.® Plasmid DNA Mini Kit II Spin Protocol (OMEGA bio-tek) for the isolation of
353 plasmid DNA.
354 6) The ON cultures were transferred to 15 mL falcon-tubes and centrifuged for 5 minutes
355 at 4000 rpm (Centrifuge 5804 R, Eppendorf).
356 7) The supernatants were removed and the pellets re-suspended in 500 µL of TE-Buffer,
357 transferred to 1.5 mL eppendorf tubes and centrifuged for 1 minute at max speed
358 (Microfuge 16, Beckman Coulter).
359 8) The supernatant was again removed and the pellet collected.
360 9) The pellet was re-suspended in 250 µL Solution I/RNase A and 10 µL mix of lysozyme
361 (40 µg/µl) and mutanolysin (1000 ug/µL) by vortexing. RNase A was added to Solution
362 I from a vial supplied by the manufacturer.
363 10) The tubes were incubated in a water bath at 37 ˚C for 10 minutes.
364 The rest of the plasmid DNA extraction was performed according to the description given by
365 the manufacturer. Lysozyme and mutanolysin were used because we wanted to isolate plasmids
366 from Gram-positive bacteria. These enzymes result in lysis of the peptidoglycan walls.

### 367 2.2.4 Preparation of Agarose Gels

368 The agarose gel slab was made with 1 g agarose mixed in 100 mL 1 % (w/v) TAE and 4 µL
369 peqGreen (VWR peqLab) in the following way:
370 1 g Ultrapure™ Agarose (Thermo Fisher) was added to 100 mL TAE buffer, and then heated
371 and solubilised in a microwave. The microwave was set to maximum effect for one minute to

372 bring the solution up to a boil, then the effect was reduced to 300 W and run for two more
373 minutes of simmering. The melted agarose solution was pre-stained with 4 µL peqGreen
374 (VWR, peqLab) while swirling gently to mix when the solution had cooled to 55 ˚C. Melted
375 pre-stained agarose solution was then poured into casts and a comb was placed at the upper
376 notch of the cast. Gel slabs were ready to use after 30 min.
377 The agarose gel method separates negatively charged DNA fragments according to size, using
378 electric current. Since DNA is negatively charged at neutral pH, the DNA will migrate from
379 the negative pole towards the positive pole in the agarose matrix. The agarose gel works by
380 letting the smaller fragments migrate faster than larger fragments. Pore sizes in the agarose
381 gel matrix are determined by its percentage of agarose. peqGreen has the same properties as
382 Ethidium Bromide, as it intercalates with DNA, making it visible under UV light.

### 383 2.2.5 Cutting of Plasmids with Restriction Enzymes

384 Before the purified plasmid DNA were screened on an agarose gel electrophoresis. The
385 plasmid DNA was cut with two restriction enzymes SpeI (New England BioLabs®) and XbaI
386 (New England BioLabs®). The cutting of plasmid DNA was performed by mixing 10 µL
387 plasmid DNA sample with 10 µL restriction enzyme master mix, which were incubated at 37
388 ˚C for 2 h, and used on the agarose gel electrophoresis or stored at -20 ˚C if not used right
389 away.
390 The procedure for making a master mix for e.g., 21 samples:
391 1. 42 µL NEBuffer CutSmart (10x), (New England BioLabs®)
392 2. 10.5 µL XbaI (20 000 U/mL), (New England BioLabs®)
393 3. 10.5 µL SpeI (10 000 U/mL), (New England BioLabs®)
394 4. 4.2 µL BSA (100x), (New England BioLabs®)
395 5. 142.8 µL $dH_2O$

### 396 2.2.6 Preparing 1 kb DNA ladder

397 A ready to use 200 µL 1 kb DNA ladder (0.05 µg/µL), aliquoted in 1.5 mL eppendorf tubes,
398 was prepared in the following way:
399 1) 20 µL of 1kb DNA ladder (0.5 µg/µL) (#N3232, New England BioLabs®)
400 2) 30 µL of 6X Loading Dye, no SDS (#B7025S, New England BioLabs®)
401 3) 150 µL $dH_2O$
402 The ready to use 1 kb ladder was mixed gently and stored at 4 ˚C.

403 ### 2.2.7 Agarose gel electrophoresis and visualisation of DNA

404 The plasmid DNA of 10 samples was run on an agarose gel electrophoresis in a 1 % (w/v)
405 agarose gel at 70 V for approximately 1.5 hours. Procedure for agarose gel electrophoresis of
406 cut plasmids were as follows: The cut plasmid DNA (20 µL) were mixed with 3 µL of 6x
407 Loading Buffer (New England BioLabs®), and 15 µL was loaded into the agarose gel wells,
408 prepared as described above. The wells on each side of the samples was loaded with 10 µL of
409 1kb DNA ladder (New England BioLabs®). The fragment size of the plasmids was compared
410 to the 1 kb DNA ladder (New England BioLabs®). The Molecular Imager® Gel Doc™ XR
411 Imaging System with Quantity One 1-D analysis software, v.4.6.7 (Bio-Rad laboratories) was
412 used for visualization of agarose gels.

413 ### 2.2.8 16S rDNA PCR and Sequencing Preparation

414 The polymerase chain reaction (PCR) amplification was performed with OneTaq® DNA
415 polymerase (New England BioLabs®), 5x OneTaq® Standard Reaction Buffer (New England
416 BioLabs®), 11F (20 µM) forward primer (primer 1), and 4R (20 µM) reverse primer (primer
417 2). For a 50 µL reaction, a master mix of 49 µL was added to 1 µL DNA template in each
418 well of a 96 well PCR plate (VWR).

419 Table 5. PCR master mix for 1 sample. 1 µL DNA template was added to 49 µL PCR master mix.

| SOLUTION | VOLUME (µL) |
|---|---|
| 5x OneTaq® Standard Reaction Buffer | 10 |
| dNTP 10 mM | 1 |
| primer 1: 11F 20 µM | 1 |
| primer 2: 4R 20 µM | 1 |
| Sterile d$H_2O$ | 35.7 |
| OneTaq® DNA Polymerase | 0.3 |

420

421 All PCR amplification of 16S rDNA were performed in a PTC-100™ Programmable Thermal
422 Controller (MJ Research), which was programmed with the following amplification
423 conditions and cycle steps:

| NUMBER OF CYCLES | AMPLIFICATION EVENT | TIME |
|---|---|---|
| **1 cycle** | Initial denaturation | 3 min at 97˚C |
| | Denaturation | 15 s at 95˚C |
| **30 cycles** | Annealing | 30 s at 55˚C |
| | Extension | 1 min, 30 s at 72˚C |
| **1 cycle** | Final extension | 10 min at 72˚C |

| ∞ | Finished | 4˚C for ∞ |
|---|---|---|

424

425 Afterwards, the correct size (ca. 1.5 kb) of amplicons was confirmed using 1% (w/v) agarose
426 gel electrophoresis at 75 V for 45 min with 1kb DNA ladder (New England BioLabs®) as a
427 size marker. The gel was prepared as described above, but with an additional comb in the
428 middle.
429 The amplified PCR product was cleaned with NucleoSpin® Gel and PCR Clean-up kit
430 (Macherey-Nagel), concentration of the DNA was determined with Nanodrop (Wilmington,
431 DE, USA), standardised to 20-80 ng/µL DNA. 5 µL was used as templates in a sequencing
432 reaction together with 5 µL 11F (5 µM) primer on a 96 well microtiter plate (VWR, USA).
433 The 96 well microtiter plate was sent to GATC Biotech AG for sequencing on their Sanger
434 ABI 3730xl sequencing machine.

### 435 2.2.9 Analysis of 16S rDNA sequences

436 The 16S rDNA sequences were analysed by downloading the ABI- files from GATC Biotech
437 AG, generated from automated Sanger sequencing (Sanger ABI 3730xl), and loading them
438 into BioEdit (v7.2.5) for editing. In the BioEdit interface, the chromatograms in the ABI-file
439 visualised the sequence, and assisted in the removal of poor sequence regions at the ends of
440 the sequence. The edited sequences were exported to fasta-files, which were used in BLAST
441 (Basic Local Alignment Search Tool) searches in GeneBank (National Centre for
442 Biotechnology Information) to search for homologues 16S rRNA gene sequences among the
443 species in the Ribosomal Database Project (RDP II), and identification. The "top hits",
444 sequences producing significant alignments, in the BLAST search were used, registering the
445 species description and accession number, maximum score, total score, query cover, E-value
446 and identity of the alignment. The identified 16S rDNA sequences were then further
447 processed and compared with various bioinformatics tools to determine phylogeny.

### 448 2.2.10 Phylogenetic analysis of 16S rDNA sequences

449 All sequences were loaded into the Bioedit program as one fasta formatted file. The ClustalW
450 Multiple Alignment program included in BioEdit was used to make a multiple sequence
451 alignment (MSA). It was created with default options, which include bootstrapping NJ
452 (Neighbor Joining) tree, number of bootstraps were 1000, a pairwise alignment of each
453 sequence to one another and calculation of a NJ tree. To construct the tree, the accessory
454 application DNADist Neighbor phylogenetic tree is run on the created MSA sequences. This
455 MSA was then used to construct a phylogenetic tree. The method used by BioEdit is the NJ

456 method of Saitou and Nei (Hall, 2013). First, distances are calculated (percent divergence)
457 between all pairs of sequence from a multiple alignment; secondly, applying the NJ method to
458 the distance matrix (Hall, 2013).
459 The "outree"-file created by the BioEdit software were loaded into the program Dendroscope
460 for editing (Huson and Scornavacca., 2016).

461 **2.2.10 Fermentation Profiling of Carbohydrate Metabolism**

462 The commercial API 50 CH kit (bioMérieux) and API 50 CHL medium (bioMérieux) were
463 used in the determination of carbohydrate fermentation of selected strains from the bee
464 isolates that previously had been analysed by 16S rDNA sequencing. 20 strains were selected
465 at random within each genus and time of collection.

466 1. The API 50 CHL medium was brought to RT before use, and the API 50 CH kit was
467 assembled according to the manufacturer.
468 2. 1 mL of ON culture of the bacteria strain was centrifuged for 5 min at 13000 rpm.
469 3. The supernatant was then removed
470 4. The cell-pellets were washed with some (100 µL) API 50 CHL medium (bioMérieux),
471 and centrifuged for 1-2 min (depending on whether the pellet was suspended or not)
472 5. The API 50 CHL medium used for washing was removed and the pellet re-suspended
473 in 2 mL API 50 CHL medium.
474 6. 300 µL of the suspension was transferred to a falcon-tube with 6 mL API 50 CHL
475 medium and mixed on a vortex (lab-dancer).
476 7. In each of the 50 tubes, circa 100 µL was filled up to the menisci of the tubes holding
477 the incubation box at an angle.
478 8. The cupules were thereafter filled with mineral oil (Sigma-Aldrich), and incubated at
479 30˚C.
480 9. Colour change in the tubes were monitored and recorded after 24h, 36h and 72h, up to
481 7 days.

482 **2.2.7 Bacteriocin screening**

483 The 190 isolates were screened for bacteriocin activity, seen as inhibition of growth around
484 the applied test-strain on a lawn of four indicator strains. The indicator strains, which are
485 listed in materials above, are *Listeria innocua*, *Lactobacillus plantarum*, *Lactococcus lactis*,
486 and *Lactobacillus sakei*.

487 The stock solutions of all isolates were streaked out on a MRS-agar plates. The indicator-
488 strains were inoculated and grown on BHI-broth. Both indicator strains and test-strains were
489 incubated ON at 30˚C.
490 Soft agar assays were carried out as a qualitative analysis to determine if there were any
491 bacteriocin activity among the different strains. All assays were carried out by diluting ON
492 cultures of indicator strains approximately 100-fold in MRS soft agar (40 µL of ON culture in
493 5 ml of MRS soft agar) and immediately plate out the solution on MRS agar plates. BHI was
494 used for some of the isolates. After air drying the plates for 5-10 min, a toothpick with each
495 test-strain were dotted or 3 µL were spotted on the lawn of indicator strains. A 2 µL spot with
496 *L. garvieae* was used as a control. The plates were then incubated at 30˚C ON. Some were
497 incubated anaerobically in jars with AnaeroGen™ 3.5L gas packs (Thermo Scientific). The
498 next day the plates were taken out of the incubator and the diameter of any zones of inhibition
499 was measured. The control strain *Lactococcus garvieae* KS1546, which produces the broad
500 spectrum bacteriocin GarKS (unpublished work at the LMG laboratory at NMBU), was
501 supplied by Kirill Ovchinnikov.
502

503 # 3. Results

504 The results from a similar work on flowers was obtained from Marte S. Linjordet (2016). Any
505 mentioning from now on regarding flower results are from the experiments made by Marte S.
506 Linjordet on flowers we collectively sampled during summer of 2015 (May – August) – at the
507 same time as the bee samples, which I have used in this thesis. Disposition to the bee gut
508 material was obtained from professor Dzung Bao Diep.

509 ## 3.1 Bacterial Flora

510 For the purpose of assessing the amount of cultured bacteria from the bee gut with respect to
511 time and discussion of species or genera found in the samples, the amount of cultured bacteria
512 has been calculated and are reported as colony forming units per mL gut microbiota sample.
513 The C3T9 sample from June, that was incubated in 30˚C ON, had 86 colonies on the -1
514 dilution plate, which is 1.6 x$10^4$ CFU/mL bee gut sample. The C3T6 sample from mid-May,
515 that was diluted and incubated in 30˚C ON and then RT ON, had 554 colonies on the -1
516 dilution plate which is 1.1 x$10^5$ CFU/mL bee gut sample. The C3T12 sample from early
517 August, that was incubated in 30˚C for two days, had 737 colonies on the -1 dilution plate
518 which is 1.4 x$10^5$ CFU/mL sample. The C3T13 sample from late August, that was diluted on
519 the 2$^{nd}$ of February and incubated for 8 days in 30˚C, had 353 colonies on the -4 dilution plate

520 which is 7.0 $x10^7$ CFU/mL sample. The -3 dilution had too many colonies to be counted
521 accurately but had to be used since the colonies picked from the -4 dilution had not yielded
522 any pure cultures after 5 days. Of the 40 pure cultures from both dilutions -3 and -4 that were
523 made, only 15 isolates were obtained from each. The C3T7 sample from late May, that was
524 diluted on the 3rd of February and incubated in 30˚C for two days, had 258 colonies on the -1
525 dilution, which is 5.0 $x10^4$ CFU/mL sample.
526 Table 6. Summary of the colony forming units (CFU) on the dilution plates described above.

| SAMPLE | TIME POINT SAMPLED | SAMPLE DILUTION | COLONY FORMING UNITS IN THE SAMPLE |
|---|---|---|---|
| C3T6 | Mid-May | -1 | 1.1 $x10^5$ CFU/mL |
| C3T7 | Late May | -1 | 5.0 $x10^4$ CFU/mL |
| C3T9 | Mid-July | -1 | 1.6 $x10^4$ CFU/mL |
| C3T12 | Early August | -1 | 1.4 $x10^5$ CFU/mL |
| C3T13 | Mid-August | -4 | 7.0 $x10^7$ CFU/mL |
| C3T13 | Mid-August | -3 | 2.7 $x10^7$ CFU/mL* |

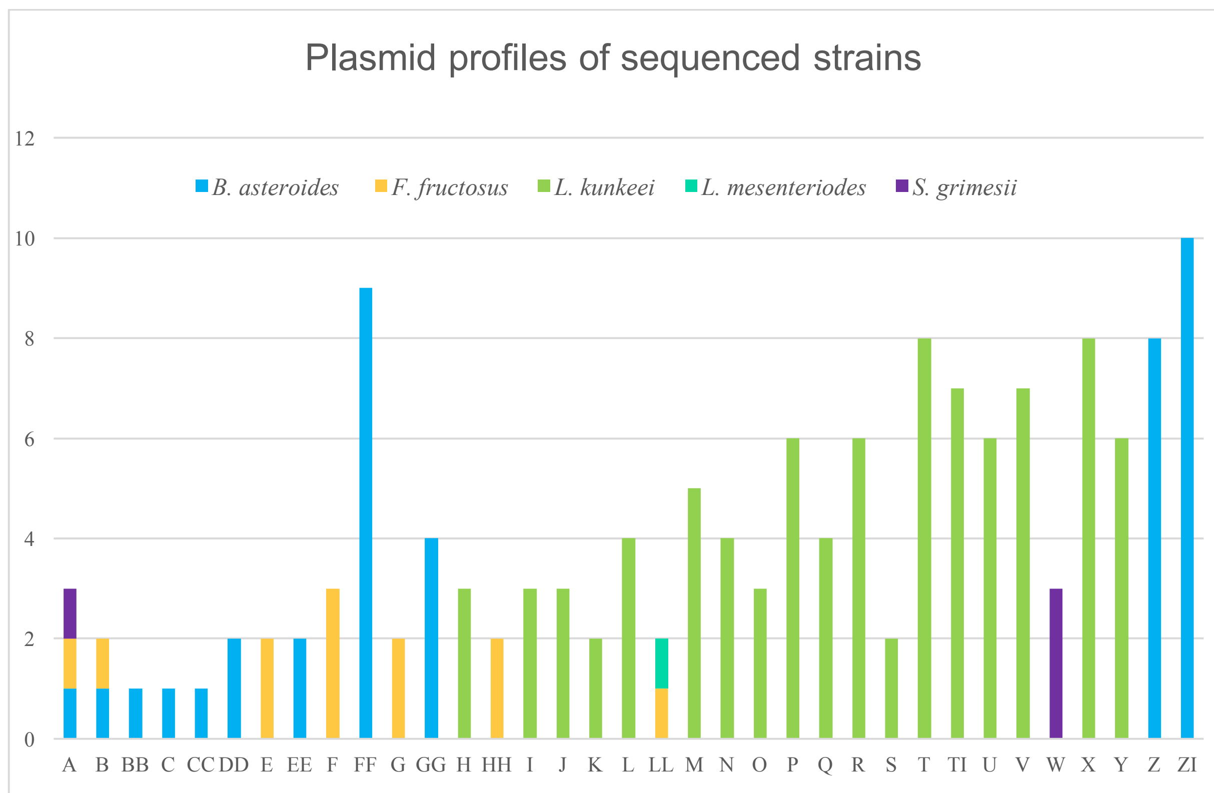
527 * Counting ca 175 colonies on 1/8 of the plate. The dilution plate (-3) was counted, and stored in the refrigerator,
528 5 days before the -4 plate which was incubated in 30˚C before use and counting.

529

530 ## 3.2 Plasmid profiling

531 The 190 isolated bee gut bacterial strains had 37 different plasmid profiles, including the
532 plasmid profile Zero given to 50 strains without plasmids. The next most abundant profile is
533 A, with 30 strains. Plasmid profile A, has one plasmid and comprises mostly strains belonging
534 to the C3T9 sample where two are identified by 16S rDNA sequencing and two strains from
535 the C3T13 sample. The third most abundant profile B has one plasmid, with 24 strains, where
536 23 of the strains belongs to the sample C3T12 and one strain belongs to C3T13.
537

538




539 Figure 2. Bar chart diagram of the plasmid profiles of identified strains. The y-axis shows the number of
540 plasmids in the plasmid profile which is given on the x-axis. The bars are coloured after which species that is
541 represented in the profile. In plasmid profile A, which have one plasmid, there are three species represented; *B.*
542 *asteroides* (blue), *F. fructosus* (yellow), and *S. grimesii* (purple). The plasmid profile LL, has one plasmid and
543 the species represented are *F. fructosus* (yellow) and *L. mesenteroides* (turquoise). The green bars are all *L.*
544 *kunkeei*.

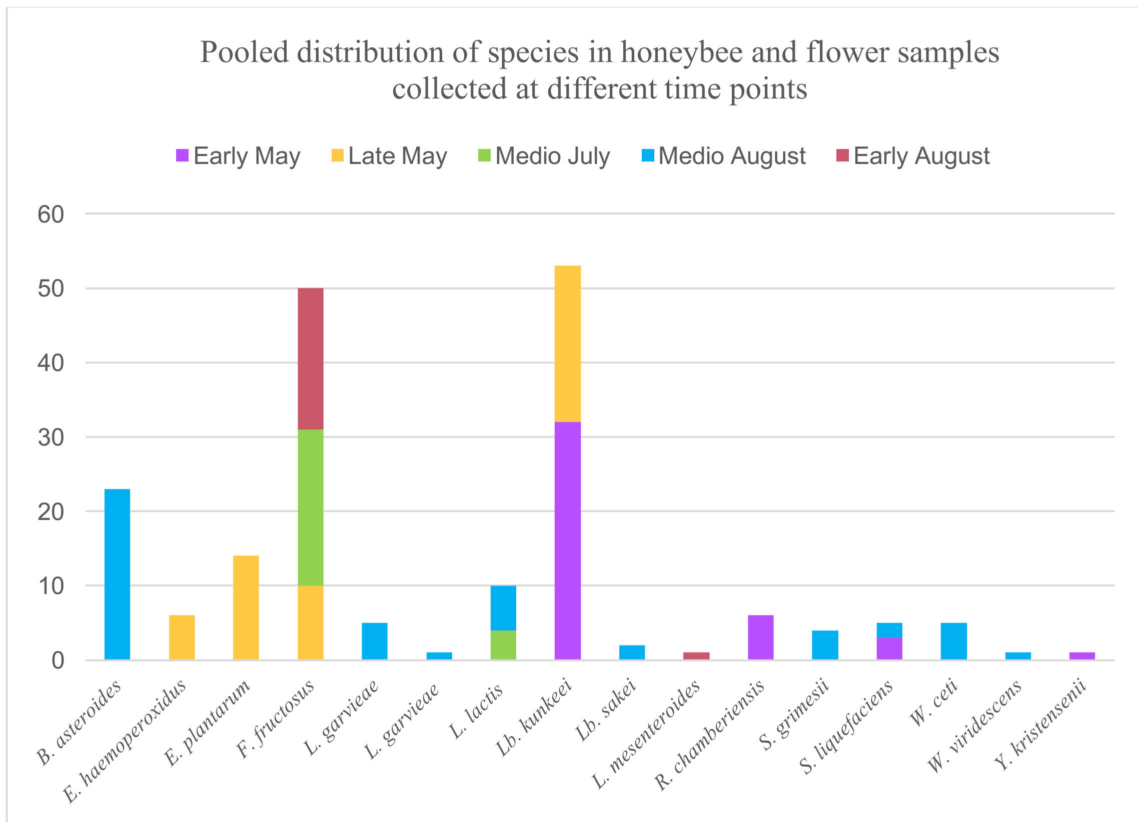
## 545 3.3 Analysis of 16S rDNA sequences

546 The analysis of 16S rDNA sequences obtained from samples sent to GATC Biotech for
547 Sanger sequencing in 96 well plates, resulted in identification of far more diverse bacterial
548 flora derived from flowers than the identified bacteria from the gut microbiota of honeybees.
549 The flower derived bacteria identified were composed of the genera *Lactobacillus*,
550 *Lactococcus*, *Weissella*, *Enterococcus*, *Yersinia*, *Rouxiella*, *Serratia*, and *Fructobacillus*. In
551 the gut microbiota of bees, strains identified, belonged to species of the genera
552 *Bifidobacterium*, *Fructobacillus*, *Lactobacillus*, *Leuconostoc*, and *Serratia*.
553 There has been identified 6 species in bee gut microbiota and 12 species in flower samples
554 during the 4 months of summer 2015. In total, 15 bacterial species have been identified in
555 bees and flowers; *Weissella ceti*, *Weissella viridescens*, *Lactococcus lactis*, *Lactococcus*
556 *garvieae*, *Lactobacillus sakei*, *Enterococcus haemoperoxidus*, *Enterococcus plantarum*,
557 *Yersinia kristensenii* and *Rouxiella chamberiensis* that were only identified in the flowers, in

558 addition to *Serratia liquefaciens*, *Fructobacillus fructosus*, and *Lactobacillus kunkeei* who
559 were also identified in gut microbiota of honeybees. *Bifidobacterium asteroides*, *Serratia*
560 *grimesii*, and *Leuconostoc mesenteroides* were only identified in the honeybee.

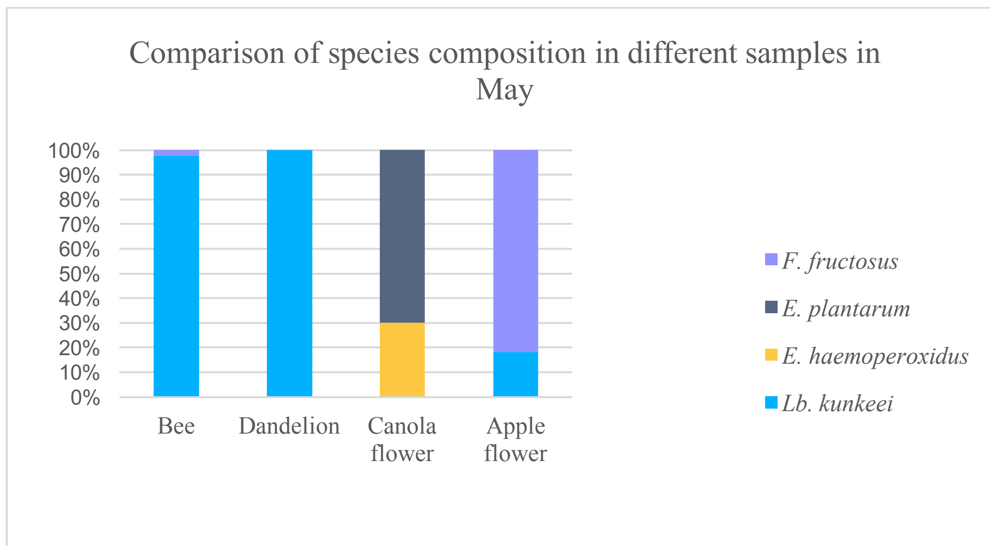
561 ### 3.3.1 Phylogeny

562 ### 3.3.1 Comparing flower and bee derived bacteria



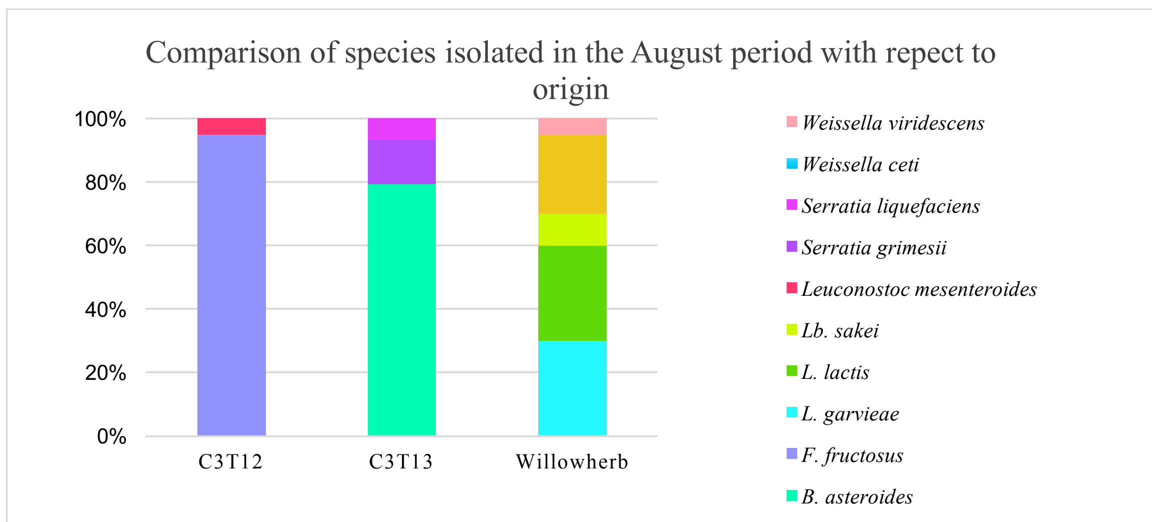

563
564 Figure 3. A stacked bar chart of all the different bacteria in honey bee samples and flower samples that has been
565 identified by sequencing the 16S rDNA. The colours in the diagram represent the different periods the samples
566 were collected, while the y-axis shows the how many strains that was identified as a given species, which are
567 given on the x-axis.

568 The comparison of bee gut microbiota in the May with LAB found in Dandelions, autumn
569 sown Canola flowers and Apple flowers. The bacterial strains that were identified in
570 dandelions and bee samples C3T6 and C3T7 were composed of *Lactobacillus kunkeei*.
571 *Lactobacillus kunkeei* was also identified in apple flower samples, but to a lesser extent than
572 in dandelions. As seen in figure 5 the Autumn sown canola flowers, however, had no
573 correlation with the identified species of any sample. The strains identified in both bee and
574 flower samples in June were all *Fructobacillus fructosus*.
575

576 


577 Figure 4. Diagram of the lactic acid bacteria identified in the samples from May.

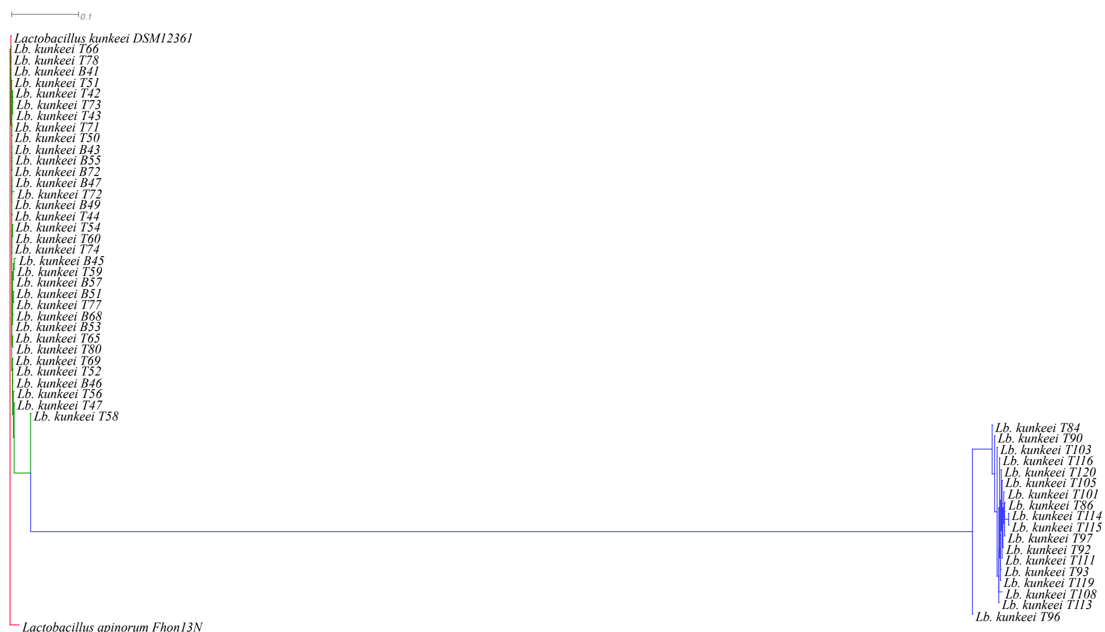
578 The aerobically grown species, *Yersinia*, *Serratia* and *Rouxiella*, from Dandelions have been
579 omitted, however, a figure with the aerobically grown species included, can be found in the
580 appendix. They were left out of the comparison between flower and honey bee gut bacteria
581 because of the different culture conditions that affect the culturable bacterial composition in
582 the samples. The anaerobic nature of the bee gut is not comparable in these settings. Although
583 *Serratia* species has been found in the bee sample C3T13 that was grown anaerobically.

584 


585 Figure 5. The August period had a more diverse bacteria flora composition than what is seen in the three
586 foregoing months. Lactobacillus kunkeei, however, is absent at this point. None of the bacteria are found in both
587 samples.

588 The bee samples from the period of early and late May was mainly composed of
589 *Lactobacillus kunkeei*. A phylogenetic tree of *L. kunkeei* identified by 16S rDNA sequencing
590 revealed that the *Lactobacillus kunkeei* from bees in late May, cluster in one of two groups.

591 The late May group seems to be phylogenetically distinct from *L. kunkeei* in C3T6 bees from
592 early May (and flowers). The BLAST analysis of all *Lactobacillus kunkeei* strains in this
593 study had high 16S rRNA gene sequence similarities to both *Lactobacillus kunkeei* (strain
594 YH-15, with accession number NR_113579.1) and *Lactobacillus apinorum* (strain Fhon13N,
595 with accession number NR_126247.1); identity and query cover ranging from 99-100%, and
596 an E-value of 0.0. This was especially true for the late May bee samples which had the same
597 query cover and identity score to both species. The early May bees, and all flowers, on the
598 other hand had higher sequence similarity to *Lactobacillus kunkeei* and to each other. As a
599 result, the phylogenetic tree in Figure 6 was constructed by means of using the type strains of
600 *Lactobacillus kunkeei* (DSM 12361) and *Lactobacillus apinorum* Fhon13N (DSM 26257) as
601 outgroups in an alignment of all *Lactobacillus kunkeei* strains.



602

603 Figure 6. Phylogenetic tree of *Lactobacillus kunkeei* strains in this study. The outgroups are
604 the type strains of *Lactobacillus kunkeei* DSM 12361 and *Lactobacillus apinorum* Fhon13N.
605

## 606 3.4 Fermentation profiling of carbohydrate metabolism

607 From the bee isolates that had been analysed by 16S rDNA sequencing 20 strains were
608 selected at random within each genus. The results from the flower microflora were also made
609 available and is included here when a comparison between the two are appropriate.

610 The *Lactobacillus kunkeei* strains T56, T60, T73, T78 (and the flower strains) from group 1 in
611 the phylogenetic tree in, and T90, T97, T103 and T115 from group 2 in the phylogenetic tree,
612 have been biochemically profiled with the API 50 CH system (bioMérieux).
613 The *F. fructosus* strains of bees that were tested was T26, T39, T117, T138, T153.

614 Table 7. Fermentation profiles of all isolated strains of fructophilic lactic acid bacteria (FLAB),
615 *Lactobacillus kunkeei* and *Fructobacillus fructosus* from bees and flowers. (*L. mesenteroides* excluded)

| API 50 CHL test | *Lactobacillus kunkeei* strains | | *Fructobacillus fructosus* strains | |
|---|---|---|---|---|
| Carbohydrate | Bee | Flower | Bee | Flower |
| D-glucose | + | + | + | + |
| D-fructose | + | + | + | + |
| D-mannitol | + | + | + | + |
| Esculin ferric citrate | d | - | -/w | - |
| D-saccharose (sucrose) | + | + | - | - |
| D-trehalose | + | + | - | - |
| D-raffinose | d- | - | - | - |
| Amidon (starch) | d- | - | - | - |
| Potassium gluconate | + | w | - | - |

616 + = 90% or more of strains are positive; – = 90% or more of the strains are negative; d = 41 – 59% of strains are positive; d-
617 =11 - 39% of strains are negative; d+ = 60 - 89% of strains are positive; w = weak reaction (i.e., light green colour, or the
618 hard to distinguish colour of black(positive) from very dark blue/brown in Esculin); -/w = one strain has a deviant weak
619 reaction as opposed to a negative reaction observed in any other strains of the same species, and that does not fit the meaning
620 of d, d+ or d-.
621

622 The fermentation profiles of LAB in July included *Fructobacillus fructosus* strains T26, T39,
623 B23, B29 and *Lactococcus lactis* strain B37, all ferment D-Fructose, D-Glucose, and D-
624 Mannitol. Lactococcus lactis also fermented Amidon (starch), Amygdalin, Arbutin, D-
625 cellobiose, Esculin ferric acid, D-galactose, Gentiobiose, D-lactose, D-maltose, D- mannose,
626 N-acetyl glucosamine, D-ribose, Salicin, D-trehalose, and D-xylose.
627 The fermentation profiles of all *Fructobacillus fructosus* strains from bees and flowers had
628 consistent fermentation profiles, except for the weak reaction in Esculin ferric acid in some
629 bee strains.
630

631 Table 8. Table of all fermentation profiles from bee strains, and that of flowers which correspond to bee species.
632 Duplicate species are merged to into one profile.

| API 50 CHL | Fermentation profiles of strains isolated from bees and selected flower strains | | | | | | |
|---|---|---|---|---|---|---|---|
| Carbohydrate | *Lb. kunkeei* | | *F. fructosus* | | *B. asteroides* | *L. mesenteroides* | *Serratia* spp. |
| | Bee | Flower | Bee | Flower | | | |
| Glycerol | - | - | - | - | d | - | d |
| L-arabinose | - | - | - | - | - | + | + |

| D-ribose | - | - | - | - | + | + | + |
|---|---|---|---|---|---|---|---|
| D-xylose | - | - | - | - | - | + | d |
| D-galactose | - | - | - | - | d | + | - |
| D-glucose | + | + | + | + | d | + | - |
| D-fructose | + | + | + | + | d | + | - |
| D-mannose | - | - | - | - | d | + | d |
| L-rhamnose | - | - | - | - | - | - | + |
| Dulcitol | - | - | - | - | - | - | + |
| D-mannitol | + | + | + | + | d- | + | - |
| Methyl-α-D-Glucopyranoside | - | - | - | - | d- | + | - |
| N-Acetyl-glucosamine | - | - | - | - | d- | + | d+ |
| Amygdalin | - | - | - | - | d- | + | - |
| Arbutin | - | - | - | - | d- | + | + |
| Esculin ferric citrate | d+ | - | -/w | - | + | + | + |
| Salicin | - | - | - | - | d+ | + | + |
| D-cellobiose | - | - | - | - | d- | + | + |
| D-maltose | - | - | - | - | d+ | + | + |
| D-lactose | - | - | - | - | d- | + | d+ |
| D-melibiose | - | - | - | - | d | + | + |
| D-saccharose (sucrose) | + | + | - | - | d | + | + |
| D-trehalose | + | + | - | - | d | + | + |
| D-melezitose | - | - | - | - | d- | - | - |
| D-raffinose | d- | - | - | - | d | + | + |
| Amidon (starch) | d- | - | - | - | d- | - | - |
| Gentiobiose | - | - | - | - | d- | + | + |
| D-turanose | - | - | - | - | d+ | + | - |
| D-tagatose | - | - | - | - | d- | - | - |
| L-fucose | - | - | - | - | - | - | d |
| Potassium gluconate | + | w | - | - | - | - | d- |
| Potassium 2-ketogluconate | - | - | - | - | d- | - | - |
| Potassium 5-ketogluconate | - | - | - | - | w | - | - |

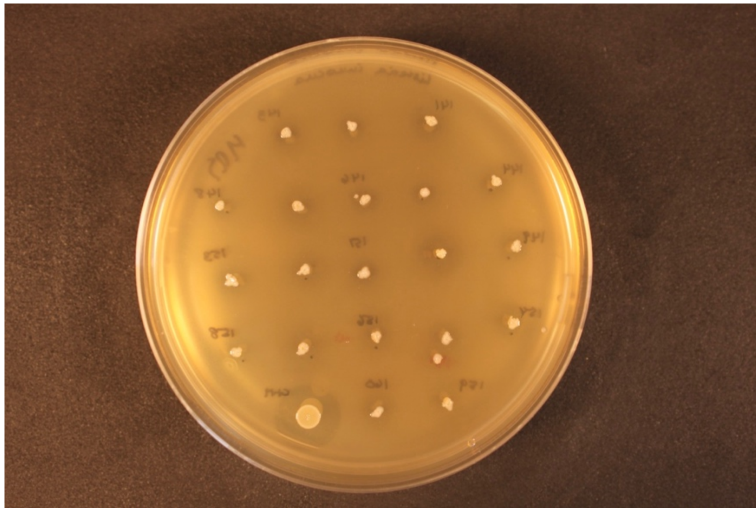
+ = 90% or more of strains are positive; – = 90% or more of the strains are negative; d = 41 – 59% of strains are positive; d- =11 - 39% of strains are negative; d+ = 60 - 89% of strains are positive; w = weak reaction

## 3.5 Bacteriocin Screening

All 190 isolated strains were proven negative for bacteriocin activity, with the method as described earlier.

In Figure 7 a photograph of the MRS plate of samples belonging to C3T12, that were screened for bacteriocin inhibiting of *Listeria innocua*.. The species in the C3T12 sample that were identified earlier was *Fructobacillus fructosus* strains and one *Leuconostoc mesenteroides.* The picture shows how the growth of *L. innocua* got inhibited by lactic acid

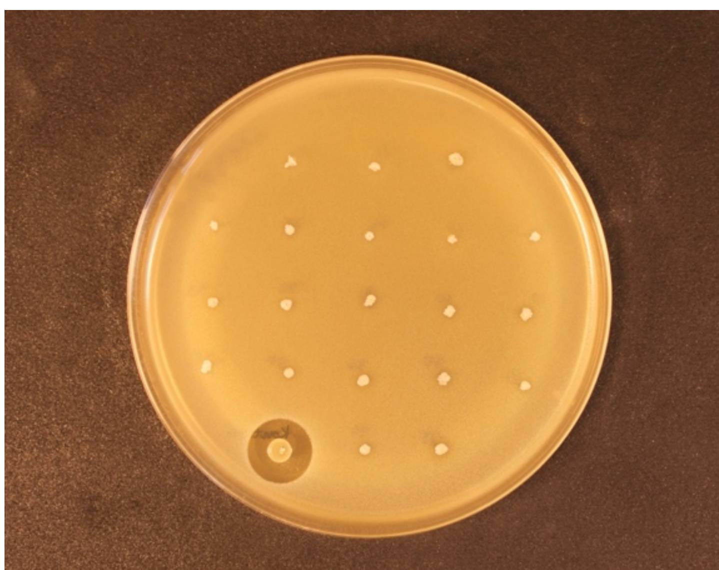
642 production, as opposed to the bacteriocin inhibition, bottom left-hand corner of the plate,
643 expressed by *Lactococcus garvieae*.



644

645 Figure 7. The diffuse area around the tested isolates on the plate with the indicator *Listeria innocua* are the result
646 of lactic acid that the test isolates produces from the sugars in the MRS medium, and not an inhibition from
647 bacteriocin like the one around the control. The control is an already known bacteriocin producing strain
648 *Lactococcus garvieae* KS1546.

649 In Figure 8, the same isolated strains as above, are screened on the indicator strain
650 *Lactobacillus plantarum*. As described earlier, bacteriocins produced by a given species are
651 usually more or only effective against closely related species. *L. plantarum*, which is lactic
652 acid bacteria closely related to *F. fructosus* and *L. mesenteroides* in the family of
653 Lactobacillaceae compared to L. innocua, did not show any inhibition of growth due to lactic
654 acid production.



655

656 Figure 8 The same isolates on a plate with *Lactobacillus plantarum* which does not get affected by the
657 production of lactic acid, as it produces lactic acid as well. The GarKS producer has a clear inhibition zone as
658 one can expect from a bacteriocin producing bacteria.

659 # 4. Discussion

660 ## 4.1 Growth of bacterial strains

661 The samples from May that was composed of Lactobacillus kunkeei, showed slow growth
662 patterns, when the diluted samples were grown in the anaerobic jars. Coincidences which led
663 to the placement of the plates on the bench ON, resulted in the formation of colonies similar
664 to those of lactobacilli. Although these were not used, new plates from the same sample was
665 made and the bacterial microbiota colonies produced, were identical. The sample was left in
666 the anaerobic jar for a longer period, over the weekend, but the anaerobic sachet was not
667 changed during this time, which in turn would have reduced the anaerobic effect in the jar.
668 As with the strains of Lactobacillus kunkeei Endo et al. (2012) these are characteristics are
669 more in accordance with fructophilic lactic acid bacteria. that are not in agreement with the
670 usual characteristics of the genus Lactobacillus, but were consistent with those of fructophilic
671 lactic acid bacteria (Endo et al., 2012).

672 ## 4.2 Plasmid profiles and 16S rDNA identification - matching

673 Plasmid profile A, as mentioned in results, has one plasmid and comprises mostly strains
674 belonging to the C3T9 sample where two are identified by 16S rDNA sequencing as
675 *Fructobacillus fructosus* and two strains from the C3T13 sample identified as
676 *Bifidobacterium asteroides* and *Serratia grimesii*. The third most abundant profile B has one
677 plasmid, with 24 strains, where 23 of the strains belongs to the sample C3T12 and one strain
678 belongs to C3T13. Six of them has been identified by 16S rDNA; five *F. fructosus* and one *B.*
679 *asteroides*. These two plasmid profiles (A and B) are very similar, having a band at 3.5 kb and
680 3.75 kb, and could in fact be the same plasmid, but, assuming that different bacteria might
681 have different plasmids the two banding patterns are assumed different. The other plasmid
682 profiles were easily recognised as different, 30 of them have more than one plasmid, and 23
683 profiles have more than two plasmids. The plasmid profiles H, I, J, K, L, M, N, O, P, Q, R, S,
684 T, TI, U, V, X and Y are all only represented by *L. kunkeei*. The ZI profile has 10 plasmids
685 and belong to *Bifidobacterium asteroides* strain T233. The species *B. asteroides* has only
686 been identified in the sample C3T13, based on the analysis of 16S rDNA and the observed
687 growth characteristics of the 24 strains, which has not been seen in any other isolate.

688 The dandelion sample was collected at Ås Campus 13th of May, two days before the bee
689 sample C3T6. The apple flower sample was collected at Ås Campus, and the canola flowers
690 were picked from a blooming canola field slightly off Ås Campus, the same day as the bee
691 sample C3T7 on 27th of May. It seems as if the canola flower bacterial flora did not have an
692 impact or correlation with the bee gut microbiota, as seen in the apple flower bacterial flora.
693
694 In total, 15 bacterial species were identified by the analysis of 16S rDNA, although the
695 Enterococcus spp., Serratia spp., Weissella spp., and some of the strains that belong to the
696 Lactococcus spp. and Lactobacillus spp., could not be reliably differentiated since sequencing
697 of the whole 16S rDNA sequence, which would require the sequencing of both forward and
698 reverse sequences and analysing the consensus of the two, was not done.
699 According to Sproer et al. (1999) in the family of Enterobacteriaceae one can divide the
700 phylogenetic relationship within the family in two groups, A and B, where group B consists
701 of two phylogenetically separate lineages, Serratia cluster I and II. Yersinia spp., Rahnella
702 aquatica, Ewingella americana and Hafnia sp., which are found in the Serrata cluster I,
703 together with the Serratia spp., that were identified in the bees. As mentioned the results from
704 NCBI were somewhat ambiguous when strains got hits to different (not written yet) genera
705 that were just as significant as the next. The addition of a new species, Rouxiella
706 chamberiensis (Le Fleche-Mateos et al., 2015), resolved some of the ambiguities when a re-
707 analysis of the sequences was done at a later stage of this work. (Le Fleche-Mateos et al.,
708 2015) found that this new species clustered closely with Rahnella and Ewingella
709 An excerpt of the Sproer et al. (1999) phylogenetic tree and the neighbour-joining tree Le
710 Fleche-Mateos et al. (2015) made combined with the lack of sequencing the whole 16S rRNA
711 gene might explain why the sequences in our analysis could not be exclusively differentiated
712 (see Figure 11 and Figure 12).

713 ## 4.3 Sugar metabolism

714 The strains of *B. asteroides* that were tested was T164, T193, T211 and T235. These had
715 different fermentation profiles, but clustered together in the phylogram. T164 and T211 were
716 able to ferment a lot of different sugars, whereas T193 and T235 could only ferment a few.
717 The only correlation between all of them were Esculin.
718 The two *Serratia* species *Serratia liquefaciens* and *Serratia grimesii* strain T236 and T217
719 respectively had slightly different fermentation profiles, except when compared to the

720 *Serratia* spp. isolated from the flowers which did not ferment of D-saccharose, D-trehalose
721 and D-melibiose.
722 The *F. fructosus* strains were the most fastidious of the fructophilic lactic acid bacteria
723 (FLAB), fermenting only 3 sugars, while *L. kunkeei* fermented 5 of the sugars. The sugars D-
724 glucose, D-fructose and D-mannitol was fermented by both species. *L. kunkeei* fermented also
725 D-saccharose (sucrose) and D-trehalose, but there was also observed a colour change in
726 potassium D-gluconate after 72 or more hours.
727 The one *Leuconostoc mesenteroides* isolate T150 were also used in the fermentation profiling,
728 and had a diverse fermentation profile. The observation that *L. mesenteroides* was able
729 ferment so many sugars, and *F. fructosus* so few have been noted by many researchers before
730 (Endo et al., 2015), and was also one of the reasons for moving *F. fructosus* out of the
731 *Leuconostoc* genus to its own, namely *Fructobacillus*. The reason might be that *F. fructosus*
732 have become auxotroph, and requires a particular additional nutrient that the non-auxotrophic
733 strains do not need.

734 **4.4 Bacteriocin screening.**

735 The qualitative analysis of bacteriocin production of bee gut microbiota derived strains,
736 showed no positive results for bacteriocin activity among all 190 strains. It might seem
737 strange that none of the strains exhibited any bacteriocin activity, however, scientists have
738 tried administering active bacteriocin to a pig via oral intake of lacticin 3147. After the oral
739 consumption they tried to recover the lacticin peptides in faecal material, the bowel and facies
740 (Villa and Vinas, 2016). This study ended in the conclusion that the lacticin probably were
741 inactivated during the passage of the stomach.
742 As mentioned in the methods 2.2.11 some bacterial strains had to be grown anaerobically. In
743 the first method, the propagation was performed on MRS-agar aerobically. This was done for
744 the first 160 isolates, and the 10 last isolates which consist of six *Serratia* and four *B.*
745 *asteroides*. With 10 available spots, the isolates T73, T101 and T103 (*L. kunkeei*), T150 (*L.*
746 *mesenteroides*), T64, T85, T102, T106 and T107 were inoculated in culture tubes with 5 mL
747 MRS-broth ON in 30˚C to confirm or disprove that the inhibition zone around their spots,
748 made with the toothpick method earlier, was the result of acid production. 3 µL of each strain
749 was spotted onto the MRS-agar surface, which is the same spot on lawn technique used for
750 the control, and incubated ON in 30˚C. For the rest of the *Bifidobacterium* the method was
751 changed to the spot on lawn method since the propagation by the streaking on MRS-agar
752 method described above were insufficient, and the sparse growth of the four bifidobacteria

753 doted on the screening plate earlier, and generally slow growth on MRS broth in ordinary
754 culture tubes which had been incubated aerobically ON in 30˚C seen earlier.
755 The *B. asteroides*, and one of the already tested *B. asteroides*, was therefore inoculated in
756 BHI broth culture tubes with screw lids that were filled to the top to get an anaerobic
757 environment, and incubated for 4 days in 30˚C. The MRS soft agar was also changed to BHI-
758 soft-agar, and the bacteriocin screening plates were incubated in anaerobic jars ON in 30˚C
759 after depositing 3 µL of test strain and 2 µL of the control strain as described above.
760

## 761 5. Conclusion

762 The results show a significant amount of lactic acid bacterial species known to be inhabitants
763 of the bee gut microbiota, as well as *Bifidobacteria.* The other species represented in the
764 samples, identified 16S rDNA, are also consider as bacteria common to the bees. The
765 comparison of identified lactic acid bacteria of isolated strains from bees and flower had
766 various amounts of agreement looking at all isolates from all four months of sampling,
767 however, in the early the summer months the microbiota was more similar. The study of
768 phylogeny, genetics and phenotypical characteristics conducted in this thesis have been
769 comprehensive. More work on bacteriocin screening, such as testing other methods, would
770 have been interesting in further work. Future work on the phylogenetic placement of
771 Lactobacillus kunkeei group 2, are especially interesting since it does not branch closely with
772 any of the type strains in phylogenetic trees produced.
773
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852

853

854

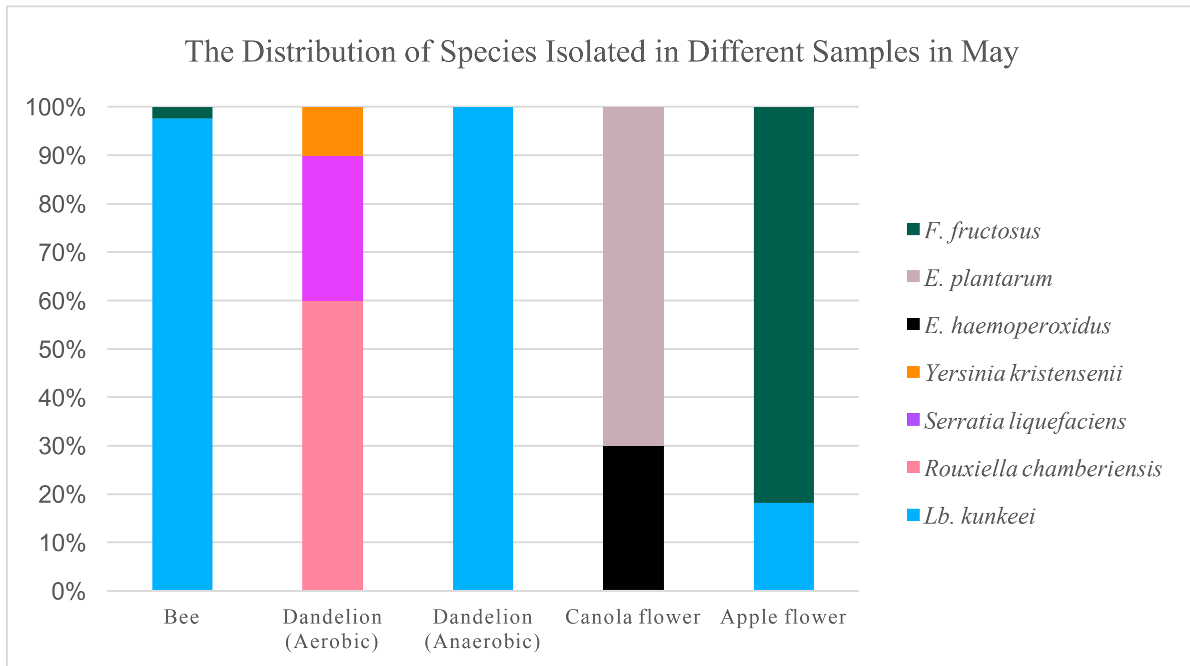
## Appendix I

Number of plasmids and strains in the plasmid profiles

| | A | B | BB | C | CC | D | DD | E | EE | F | FF | G | GG | H | HH | I | J | K | L | LL | M | N | O | P | Q | R | S | T | TI | U | V | W | X | Y | Z | ZI |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 7 C3T7 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | 7 | | | | | |
| 1 C3T7 | | | | | | | | | | | | | | | | | | | | | | | | | | | | 8 | 7 | 6 | | | 8 | | | |
| 3 C3T7 | | | | | | | | | | | | | | | | | | | | | | | | | | | 2 | | | | | | | | | |
| 2 C3T7 | | | | | | | | | | | | | | | | | | | | | | | | | 4 | | | | | | | | | 6 | | |
| 5 C3T7 | | | | | | | | | | | | | | | | | | | | | | | 3 | 6 | | 6 | | | | | | | | | | |
| 2 C3T12 | | | | | | | | | | | | | | | | | | | | 1 | | | | | | | | | | | | | | | | |
| 2 C3T6 | | | | | | | | | | | | | | | | | | | 4 | | | 4 | | | | | | | | | | | | | | |
| 8 C3T6 | | | | | | | | | | | | | | | | | 3 | 2 | | | | | | | | | | | | | | | | | | |
| 1 C3T12 | | | | | | | | | | | | | | | 2 | | | | | | | | | | | | | | | | | | | | | |
| 3 C3T6 | | | | | | | | | | | | | | 3 | | 3 | | | | | 5 | | | | | | | | | | | | | | | |
| 3 C3T9 | | | | | | | | | | 3 | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 C3T9 | | | | | | | | 2 | | | | 2 | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 C3T9 | | | | | | 3 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 C3T13 | | | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 1 C3T13 | | | 1 | | 1 | | 2 | | 2 | | 9 | | 4 | | | | | | | | | | | | | | | | | | | 3 | | | 8 | 10 |
| 23 C3T12 & 1 C3T13 | | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| 28 C3T9 & 2 C3T13 | 1 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

Figure 9. Bar chart diagram of the plasmid profiles in the bacteria isolated from honeybee gut microbiota. The isolates originating from the same sample are shown in similar colour in the figure. Profiles are indicated on the x-axis. The y-axis shows how many plasmids a plasmid profile has. In the lower part of the figure, a table of the graphical representation is shown. The number of strains within a profile (e.g., 7 C3T7, which means seven strains in sample C3T7) are indicated at the left-hand side and the number of plasmids are found in the intersection between the profile and sample in the table.

864 Appendix II




865

866 Figure 10. A stacked bar chart illustrating the comparison of all bacterial species identified in bee gut microbiota
867 and flower microflora found in all samples from May.

868

869 Appendix III

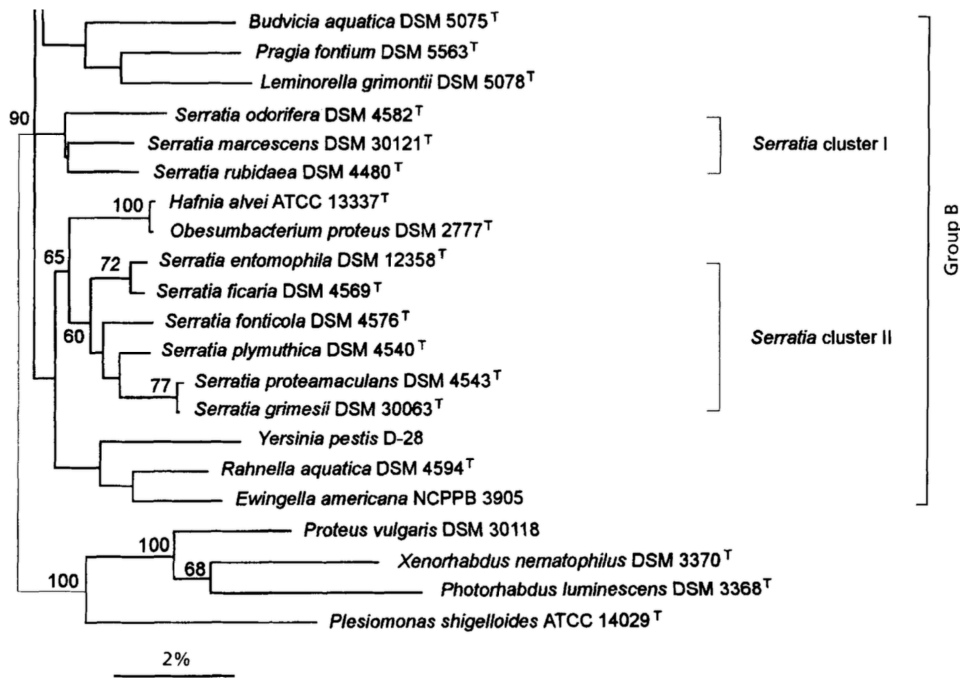


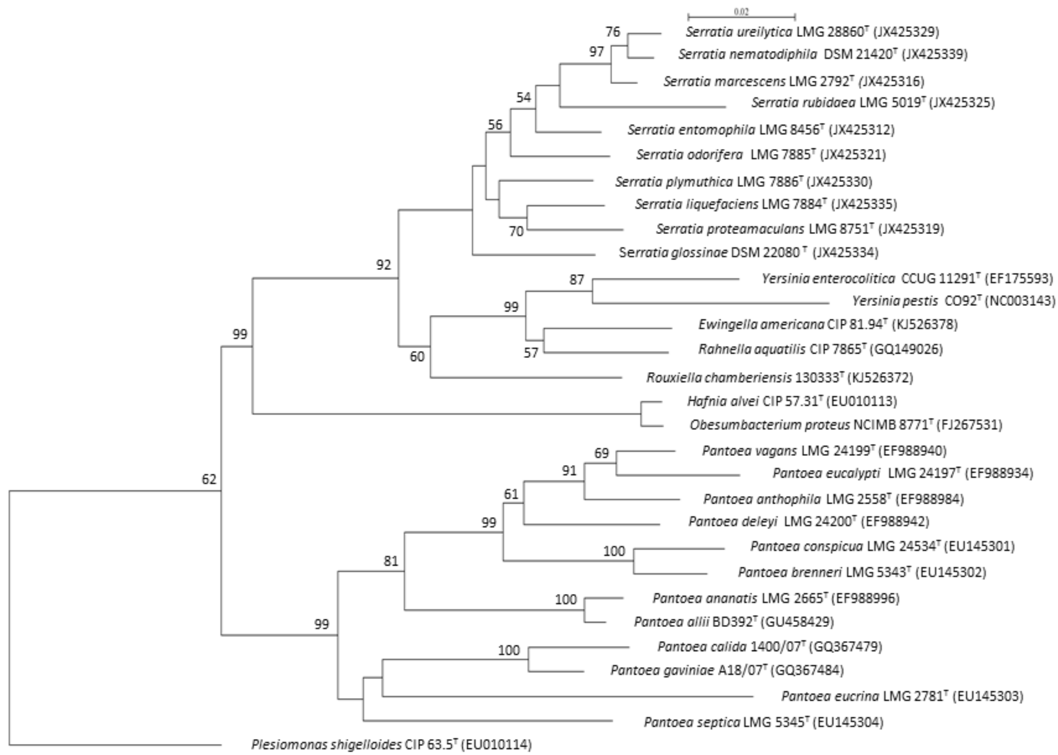

***Fig. 1.*** Phylogenetic tree of the 16S rDNA of members of various genera of the family *Enterobacteriaceae*. The positions of *Erwinia* clusters I (*Pantoea*) to IV (Kwon *et al.*, 1997) in group A and the *Serratia* clusters I and II in group B, defined in this study, are indicated. A detailed analysis of the *Buttiauxella agrestis* lineage is shown in Fig. 2. Numbers within the dendrogram indicate the occurrence (%) of the branching order in 200 bootstrapped trees (only values of 60 and above are shown). The scale bar represents 2 nucleotide substitutions per 100 nucleotides.

870

871 Figure 11. Excerpt of the phylogenetic tree of Sproer et al. (1999).

872

873 Appendix IV




874

875 Figure 12. The supplemental figure from the article Rouxiella chamberiensis gen. nov., sp.
876 nov., a new Enterobacteriaceae isolated from parenteral nutrition bags; Fig. S1. Neighbour-
877 joining tree based on rpoB gene sequences. Bootstrap values >50% (based on 1000 replicates)
878 are given at branching points. GenBank accession numbers are given in parentheses. The
879 distance scale indicates the proportion of substitutions per nucleotide position.(Le Fleche-
880 Mateos et al., 2015)

881