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A Comparative Analysis of Lactic Acid Bacteria Isolated from Honeybee Gut and Flowers, with Focus on Phylogeny and Plasmid Profile

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Abstract

Apis mellifera (honeybee) are of huge value as they are the most important pollinator worldwide. Declines in honeybee populations have made the honeybee subject to much scientific research. Lactic acid bacteria (LAB) have been discovered in the honeybee gut and are believed to be of great importance to the honeybee health, protecting them against bee pathogens. Comparing LAB communities in the honeybee gut to those found on flowers may help highlight the route and importance of floral transmission.

Using cultivation techniques selective for *Lactobacillus*, plasmid and fermentation profiling, and 16S rDNA sequencing, LAB were isolated from dandelion, apple, rapeseed, raspberry and willowherb and compared to the LAB isolated from honeybee guts.

The results showed that *Lactobacillus kunkeei* and *Fructobacillus fructosus* were the most abundant of all the identified species in both honeybee gut and flowers samples. The LAB flora of the honeybee gut seems to shift from *L. kunkeei* to *F. fructosus* through May to late June, and *F. fructosus* was also found as the most abundant LAB in one of the samples collected from honeybee guts in August. This is in accordance with the LAB flora found on dandelion and apple in May and raspberry in late June, giving indications toward a positive correlation between the LAB microbial flora in honeybee gut and the flowers within their foraging area. The results also showed the plasmid profiles were more diverse in the honeybee gut samples, and although there seemed to be four profiles that also occurred in the flower samples, indicating possible relatedness on strain level, the data sets are relatively small and further investigations are needed.

Sammendrag

Apis mellifera (honningbie) er av stor verdi og er den viktigste pollinatoren over hele verden. Reduksjoner av honningbiens populasjon har gjort at honningbien er underlagt mye vitenskapelig forskning. Melkesyrebakterier har blitt oppdaget i tarmen til honningbien og antas å være av stor betydning for honningbiens helse, hvor de beskytter dem mot bie-patogener. Sammenligning av melkesyrebakteriene i honningbiens tarm og de som finnes på blomster kan kanskje hjelpe til med å markere veien og viktigheten av bakteriell blomsteroverføring. Ved hjelp av dyrketeknikker som er selektive for *Lactobacillus*, plasmid- og fermenteringsprofilering, og 16S rDNA sekvensering, ble melkesyrebakteriene isolert fra løvetann, eple, raps, bringebær og geitrams og sammenlignet med melkesyrebakteriene isolert fra tarmen til honningbien.
Resultatene viste at *Lactobacillus kunkeei* og *Fructobacillus fructosus* var de mest tallrike av alle de identifiserte artene i både honningbietarmen og blomsterprøvene. Melkesyrebakteriefloraen i tarmen ser ut til å skifte fra *L. kunkeei* til *F. fructosus* gjennom mai til slutten av juni, og *F. fructosus* ble også funnet som den mest tallrike melkesyrebakterien i en av prøvene samlet inn fra bie-tarmer i august. Dette er i samsvar med floraen som ble funnet på løvetann og eple i mai og på bringebær i slutten av juni, noe som gir indikasjoner mot en positiv korrelasjon mellom melkesyrebakteriefloraen i tarmen og blomstene i bienes pollineringsområde. Resultatet viste også at plasmidprofilene var mer variert i honningbieprøvene, og selv om det så ut til å være fire profiler som også opptrådde i blomster prøvene, noe som kan indikere slektskap på stammenivå, var datasettene relativt små og videre undersøkelser er derfor nødvendig.

Abbreviations

CHX	Cycloheximide
FLAB	Fructophile Lactic Acid Bacteria
GC	Gas Chromatography
GI	Gastrointestinal
LAB	Lactic Acid Bacteria
MRS	De Man-Rogosa-Sharp
NMBU	Norwegian University of Life Sciences
ON	Overnight
PCR	Polymerase Chain Reaction
RT	Room Temperature

Contents

1. Introduction

Apis mellifera (honeybee) are of huge value, not only for their production of honey, but more importantly, because they are the most important pollinator worldwide. Recent declines in both wild and domestic pollinators have been a major concern and have made the honeybee subject to much scientific research.

The gastrointestinal tract in both humans and animals is known to harbor a collection of microorganisms called the gut microbiota. By extensive research on the human gut microbiota, there is evidence that the gut bacteria influence human and animal physiology, metabolism, nutrition, and immune function (1). The composition of the gut microbiota is subject to dynamic changes during the course of the host´s development, physiological status or health. For example, different gastrointestinal (GI) disorders are often linked with degenerating changes to the gut microbiota. Correspondingly, several recent studies have investigated the gut microbiota of honeybees as a response to the alarming decline in pollinators (2).

Honeybees harbor a number of commensal or beneficial bacteria distributed throughout the different compartments of their GI tracts. The GI tract of an adult honeybee is divided into four major compartments: crop (honeystomach), midgut, ileum and rectum. Each compartment has a distinct environment favoring specific microorganisms (3). Several findings have indicated that the honeybee gut is colonized by a distinctive set of bacterial species designated as the core gut microbiota (4). Because the community composition has shown to change through the bee´s life cycle, the colonization of the gut is believed to be influenced by the honeybee´s age (3). During the course of their lifespan, honeybee workers perform many different tasks that can contribute to these variations. Newly emerged worker bees nurse larvae within the hive whereas older worker bees build and maintain the wax combs, defend the colony, and receive and process food that is collected by foragers. Foragers are specialized worker bees and their job is to bring back nectar and pollen from different flowers they visit during the season.

In addition to the core microbiota in the gut, a novel lactic acid bacterial (LAB) flora composed of 13 taxonomically well-defined *Lactobacillus* and *Bifidobacterium* species

were discovered in the honey stomach of honeybees (5, 6). The honey stomach functions as an inflatable bag that can transport the nectar back to the hive for storage and honey production. It is hypothesized that LAB play a key role in the conversion of both nectar to honey and pollen to beebread (stored food rich in protein) due to their fermentation properties (5, 7) The LAB microbiota is, besides the importance in bee food processing, believed to be of great importance to the honeybee health, protecting them against bee pathogens (8, 9) and contributing to the antimicrobial properties of honey (10).

LAB are Gram-positive, usually non-motile rods or cocci that do not form spores and produce lactic acid as their major or sole fermentation product. They require environments rich in sugars, amino acids, nucleic acid derivatives, minerals and vitamins (11). LAB reside in a diversity of different habitats that includes the gastrointestinal tract of humans and animals, as well as in a number of other environments such as plants and different processed food products. LAB are found as commensals within humans, animals and insects. They are considered as beneficial organisms commonly found in healthy individuals by protecting their hosts through antimicrobial metabolites (12). Many LAB species are also indispensable to the food and dairy industry because of their key role in in the formation of taste, texture and preservation effects due to the production of antimicrobial peptides such as bacteriocins, propionic acid and other organic acids that lowers the pH (11).

LAB are found in two distinct phyla: *Firmicutes* and *Actinobacteria*. The most important genera of LAB within the *Firmicutes* are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pedicoccus*, *Streptococcus* and *Weissella,* which all have a low G+C content. LAB in the *Actinobacteria* phylum only includes species of the *Bifidobacterium* genus that in contrast to the *Firmicutes* members have a high G+C content. (13, 14)

Fructophilic LAB (FLAB) are a special group of LAB that prefers fructose instead of glucose as growth substrate (15). *Fructobacillus* spp. and *Lactobacillus kunkeei* are representatives of these microorganisms. In previous studies, *L. kunkeei* has been found to be one of the most predominant LAB in honeybees (16, 17). FLAB are found in fructose rich niches and have – in addition to honeybees – been isolated from beehives, fruits, and flowers (15, 16, 18).

Flowers are one of the many diverse habitats provided by plants for microorganisms. The availability of nutrients differs between plant parts such as roots, leaves, flowers and fruits, and within plant organs (19), and therefor contributes to varying bacterial communities. The bacterial communities colonizing roots and leaves have been the most studied for many plant species. Only recently, there have been studies on the bacterial composition of floral nectar, which initially was considered as not suitable as bacterial habitats due to their antimicrobial properties (20). Studies found that bacteria are common inhabitants of floral nectar, and that the bacterial communities differed between plant species and were characterized by low species richness and moderate phylogenetic diversity (21, 22). For bees, the floral nectar is the main source of carbohydrates and energy ('fuel'), while pollen provides proteins, lipids, vitamins and minerals for brood rearing and development (23).

Specialized honeybee worker types, nectar foragers, can learn flower attributes such as shape, color and odor, called pollinator syndromes and use this information to select for a particular flower. The individual honeybee tends to stick to one type of flower over a certain period of time(24), and can also discriminate between small differences in nectar concentration. The effect the bacterial communities may have on the floral nectar they colonize is mainly unexplored. Good et al. (25) hypothesizes that bacteria can alter the chemical profile of the nectar, thus affecting the nectar attractiveness to pollinators such as honeybees. In addition, bacteria found in floral nectar are frequently found in the honeybee hive environment and alimentary tract (26), raising questions concerning the transmission effect between flowers and pollinators visiting them for their nectar and pollen.

Foods shape the gut microbiota in animals and insects. The gut microbiota in honeybees has previously been shown to change in a season-dependent manner (27). Our hypothesis is that this can be related to the type of food the honeybees have access to along the axis of time. During the winter season honeybees in Norway still have their pollen stores, but receive artificial nectar from the beekeepers to produce honey, as no flowers are available. During foraging season (spring and summer) honeybees fly out and collect nectar and fresh pollen directly from flowers. The biochemistry and

complexity of food, and the microbial flora associated in foods, are therefor quite different between winter season and foraging season, and may likely affect the gut microbiota in honeybees. LAB are frequent in vegetables and fruits, and are also common inhabitants in gut microbiota of most vertebrates and invertebrates. The aim of this MSc thesis is to characterize LAB in selected flowers on or near campus of the Norwegian University of Life Sciences. This may contribute to a better understanding of how plant LAB are related to those present in honeybees.

Consequently, the aims of the work of this MSc-project is to:

1. Isolate and characterize lactic acid bacteria from selected spring, summer and autumn flowers using PCR, plasmid- and fermentation profiling
2. Compare the results with respect to lactic acid bacteria found in honeybee gut sampled in parallel with the flower collection

2. Materials and Methods

2.1 Sampling of Bacteria from Flowers

Flowers were collected from different locations on and near campus at the Norwegian University of Life Sciences, Ås, Norway. To represent different nectar secretions and relevant species for the production of honey in the district, flowers were collected from five plant species: dandelion (*Taraxacum officinale*), rapeseed (*Brassica napus*), willowherb (*Chamerion angustifolium*), apple (*Malus*) and raspberry (*Rubus idaeus*) (Table 2.1).

Table 2.1 Nectar production and importance for the development of honey from some plant species in the district surrounding the University of Life Sciences, Ås, Norway.

Plant species	Nectar production	Importance for the honey development in the district	Blooming
Dandelion	Medium	Important for the development	May – June
Fruit	Medium – large	Important for the development	May – June
Raspberry	Very large	Crucially important 70-90% of the honey	June-July
Autumn Rapeseed	Medium – large	Small	May
Willowherb	Large	Small	July - August

To reduce contamination, flowers were picked using rubber gloves and transported in plastic bags back to the laboratory the same day. There, they were cut with a scalpel under sterile conditions. and transferred to two 50 ml Falcon tubes before the addition of 30 ml 0.9 % NaCl in each tube. The number of flowers in each tube was 5, 20, 30, 50 and 110 for dandelion, apple, rapeseed, willowherb and raspberry respectively, to adjust for different flower mass/volumes as they ranged greatly in size.

The tubes were mixed vigorously on a vortex before the liquid containing the bacteria from each tube was transferred to two new 50 ml Falcon tubes. After centrifugation at 5000 g for 5 minutes (Eppendorf 5804 R) the pellet in one tube was suspended in 4 ml 20 % glycerol and then mixed thoroughly with the pellet in the other tube. The bacterial suspension was distributed on four 2 ml micro tubes (Sarstedt), 1 ml in each tube, and stored at -80 °C.

2.2 Growing of Bacterial Strains

The medium and the anaerobic conditions set were chosen to favor the growth of LAB. The bacterial flower samples were thawed at room temperature (RT) and 100 µl were serially diluted in 0.9 % NaCl within the range 10^{-1} – 10^{-6}.

Volumes of 100 µl were plated out on De Man-Rogosa-Sharp (MRS) agar plates and incubated at 30 °C in an anaerobic chamber until visible colonies were formed, generally within 72 hours. The chamber contained an AnaeroGen sachet (Thermo Scientific) for the generation of anaerobic conditions.

For samples that showed no colonies on the agar plates within 72 hours, a new round of cultivation was performed with either non-diluted or concentrated bacterial flower samples. For concentrated bacterial flower samples, 500 µl of the sample was centrifuged on maximum speed for 1 minute in a table centrifuge before the supernatant was discarded. The cell pellet was re-suspended in 200 µl 0.9 % NaCl before plated out on new MRS agar plates and incubated anaerobically.

For pure culture isolation, 40 colonies were picked within a random and limited area on the agar plate, and streaked on new MRS agar plates and incubated anaerobically as described above. Samples that got fewer colonies than 40 were left at RT for aerobic growth for 48 hours in an attempt to yield more isolates.

Culture tubes containing 6 ml MRS broth supplemented with 40 % fructose were inoculated with single colonies from the pure cultures using sterile toothpicks. The culture tubes were then incubated at 30 °C for 24 to 72 hours for the isolation of plasmids described in section 2.3.

For storing samples, glycerol stocks were made from pure cultures grown in MRS + fructose by adding 400 µl 45 % Glycerol to 800 µl bacterial culture in a 2 ml micro tube. The stocks were stored at -80 °C until further use. See Appendix 1 for description of preparation of MRS + fructose.

2.3 Plasmid Profiling

Plasmid profile analysis has been applied in clinical studies to investigate the spread of antibiotic resistance, where patterns of plasmids are compared between disease

outbreak strains and non-outbreak control strains (28). Plasmids are first separated according to size by agarose gel electrophoresis, and then cleaved by restriction endonucleases generating multiple fragments that make interpretation of strain relatedness more feasible (29).

2.3.1 Isolation of Plasmids

Plasmids were isolated using the E.Z.N.A. Plasmid DNA Mini Kit I (Omega) according to the protocol provided by the manufacturer with minor modifications described in the following.

Pure cultures grown in MRS + fructose overnight (ON) were centrifuged at 4000 rpm for 5 minutes before the pellet was re-suspended in 500 µl TE (Tris-Cl EDTA) - buffer. The suspension was transferred into a 1.5 ml micro centrifuge tube followed by another centrifugation step at maximum speed for 1 minute in a table centrifuge. Solution I was added together with 5 µl lysozyme (40 mg/ml) and 5 µl mutanolysine (1000 U/ml) to weaken the cell walls, and incubated on water bath at 37 °C for 10 minutes before resuming to the protocol. Bound DNA was eluted twice with 30 µl Elution Buffer and the DNA samples were kept at -20 °C until phylogenetic identification (section 2.4). A step-by-step protocol is provided in Appendix 3.

2.3.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for detection and separation of DNA according to molecular size at various stages throughout the study.

To prepare two 1% agarose gels, a mixture of 1 g agarose and 100 ml 1X TAE (Tris-acetate-EDTA) buffer was heated in a microwave oven until the agarose had dissolved. The agarose solution was cooled down to below 60 °C before 4 µl Peq-Green (VWR Peqlab) was added as a fluorescent dye to make the DNA visible when examined under UV-light. The solution was mixed by gently swirling the flask. The solution was poured evenly into two molding trays that held combs for the formation of wells and allowed to cool until the gels solidified. The solid gels were placed in electrophoresis chambers (BioRad) and the chambers were filled with 1X TAE Buffer.

Small volumes, generally 5 or 15 µl, of the samples were mixed with the appropriate amount of 6X Loading Buffer containing a visible dye and loaded into the wells on the

gel. Five or 10 µl 1kb Ladder from New England Biolabs were provided as a molecular size marker on both sides of the gel. The gels were then run at 75 V until the loading dye had traveled about 2/3 of the length of the gel. DNA bands on the gels were visualized by UV-light using a Gel Doc imager from BioRad.

2.3.3 Restriction Endonuclease Cutting

Restriction endonucleases are enzymes that recognize specific nucleotide sequences known as restriction sites and cleave the target DNA at defined positions at or near these restriction sites. To make plasmid profiles, restriction enzymes were used to cut plasmids into smaller fragments that would make a unique band pattern when run on an agarose gel.

During the pilot study, two enzymes, XhoI and SpeI, with different recognition sites were tested for their ability to cut the isolated plasmids. They were tested both together and separately. Because XhoI performed poorer than SpeI, a decision was made to replace XhoI with XbaI. This decision was based on the difference in G+C content of the recognition site. Lactic acid bacteria have a low G+C content and XhoI has a higher G+C content than both SpeI and XbaI, see Appendix 1; Table A1.1.

Reaction mixtures were prepared, while on ice, according to Table 2.2. The components were mixed gently before a short centrifugation to get all the components at the bottom of the tube, and then incubated at 37 °C for 2h. After incubation, the samples were run on a 1 % agarose gel according to the procedure described in section 2.3.2.

Table 2.2 Reaction setup for endonuclease cutting showing the components with their corresponding volumes and final concentrations for a 20 µl reaction

Component	Volume [µl]	Final concentration
10X CutSmart Buffer	2.0	1X
20 000 units/ml XbaI	0.5	10 units/20 µl reaction
10 000 units/ml SpeI	0.5	5 units/20 µl reaction
dH_2O	7.0	
Plasmid DNA	10	

2.4 Phylogenetic Identification

2.4.1 Polymerase Chain Reaction (PCR)

For phylogenetic identification, 16S rRNA gene amplification by PCR was performed on a selection of the collected bacterial strains using the universal primers 11F: (5´-TAA CACA TG CAA GTC GAA CG-3´) and 4R: (5´-ACG GGC GGT GTG TRC-3´). The DNA samples from the isolation of plasmids (section 2.3) were used as DNA templates. The reaction setup shown in Table 2.3 and the thermocycling conditions shown in Table 2.4 were applied.

Table 2.3 Reaction setup for PCR showing the components with their corresponding volumes and final concentrations for a 50 µl reaction.

Component	Volume [µl]	Final Concentration
20µM Forward Primer	1	0.4 µM
20µM Reverse Primer	1	0.4 µM
5X PCR Standard Reaction Buffer	10	1X
10 mM dNTP mix	1	0.2 mM
5000 units/ml Taq DNA Polymerase	0.3	1.5 units/50 µl reaction
dH_2O	35.7	
Template DNA	1	

Table 2.4 Thermo-cycling conditions of PCR for 16S

Steps	Temperature [°C]	Time [s]	Cycles	Description
Step 1	95	30	1	Initial denaturing
Step 2	95	15	30	Denaturing
	54	60		Primer annealing
	72	90		Primer extension
Step 3	72	300	1	Completion

In order to detect the presence of amplicons, 5 µl of the PCR product of every sample were run on a 1 % agarose gel according to the procedure described in section 2.3.2. Samples containing amplicons were purified using the NucleoSpin® Gel and PCR Clean up kit (Macherey-Nagel GmbH, Düren, Germany) according to the manufacturers instructions, and the DNA concentration was determined using a NanoDrop 2000 Spectrophotometer from Thermo Scientific.

2.4.2 Sequencing and Processing of Data

Purified PCR products were, if necessary, diluted with sterile dH_2O to the final DNA concentration of 20 to 80 ng/µl. Volumes of 5 µl were then added to a 96-well skirted tray containing 5 µl 5µM primer 11F in each well. Sequencing was performed by GATC Biotech (Constance, Germany).

The generated sequences were edited using BioEdit v7.2.5 Sequence Alignment Editor (Ibis Biosciences, Carlsbad, CA, USA). Blast analysis was performed on the edited sequences and aligned using the freely available multiple sequence alignment program MAFFT. The phylogenetic tree constructed by MAFFT was used to pick isolates for fermentation profiling based on their clustering in order to get a random sampling.

2.5 Sugar Fermentation Profiling

Twenty isolates were chosen for carbohydrate fermentation profiling using the API 50 CHL identification system from BioMèrieux, Inc. The API 50 CH strip consists of 50 microtubes that contain one negative control and 49 different substrates belonging to the carbohydrate family and its derivatives. The API 50 CH strip is used in conjunction with API 50 CHL medium that rehydrates the substrates and that contains a pH indicator that detects the production of acids when the substrate is fermented.

Sterile toothpicks were used to scrape a small amount off of the top of the freezing stock of each isolate and dropped into culture tubes with 5 ml MRS medium. The isolates were cultured ON and 1 ml of the ON-culture was centrifuged for 5 minutes at 13000 rpm. The supernatant was discarded and the pellet surface was washed with a small amount of 50 CHL medium. The cell pellet was re-suspended in 2 ml of 50 CHL medium before 300 µl of the suspension was transferred to 6 ml 50 CHL medium and mixed thoroughly. The API carbohydrate fermentation strips were prepared by placing them in the incubation tray and the bacterial suspension was distributed into the 50 tubes. Mineral oil was used to fill the cupule to create anaerobic conditions and the incubation tray was placed in a plastic box with moistened paper and incubated at 30 °C. Color changes due to the production of acids in the cupules were recorded during the course of one week.

2.6 Cycloheximide

Cycloheximide (CHX) is a eukaryotic translation inhibitor and is the most common laboratory reagent used to inhibit protein synthesis. CHX was therefore used to determine whether some of the colonies grown aerobically were yeasts.

Two isolates of in all 23 that were suspected to be yeast were inoculated in 5 ml MRS broth ON. One isolate that was proven to be *Lactobacillus kunkeei* was also inoculated in 5 ml MRS broth to function as a control.

To make a CHX stock solution, 0.157 g CHX was dissolved in 6 mL sterile dH_2O by pipetting the suspension whilst immersed in hot water. The solution was then filtrated through Filtropur S 0.2 syringe (Sarstedt) filter by the use of a 10 ml syringe.

To achieve a working concentration of approximately 5 mg/ml, 200 µl of the stock solution was diluted in 800 µl sterile dH_2O.

Five culture tubes each containing 5 ml MRS broth were prepared for each isolate, and the working solution of CHX was added in various volumes to achieve various final concentrations, see Appendix 1; Table A1.2.

The culture tubes were then thoroughly mixed before 50 µl of ON-cultures of each isolate were added to their respective tubes. The culture tubes were again mixed before incubated at 30 °C and cell density was measured on a Ultraspec10 Cell Density Meter from Amersham Biosciences after 24 hours.

3. Results

3.1 Growing of Bacterial Strains and Plasmid Profiling

Typical colonies from all different flower samples grown on MRS agar plates were white, smooth and varied little in size. Apple and dandelion samples grown aerobically were bigger in size and had rough colony edges. The duration of incubation varied between the different bacterial flower samples, and MRS agar plates were left at RT in an attempt to yield more isolates from both apple and dandelion samples. In addition, isolates from apple produced a red pigment around the edges of colonies on the periphery. Later on, these isolates were confirmed to be yeast and are therefore excluded from further estimations done on the total set of isolates.

3.2 Plasmid Profiling

One hundred and seventy-seven isolates were isolated from flowers, 40 from each plant species, except for apple samples with a yield of 17 isolates only. All isolates were assessed for plasmid content. Of the 177 isolates, 37 had plasmids, which accounts for approximately 21 %.

The majority of the isolates that contained plasmids were collected from raspberry. The results from raspberry samples are represented in Figure 3.1 containing pictures of all the agarose gels that were run. Some lanes had distinctive bands and others where only smears of DNA. Different band intensities made it difficult to obtain a single image of all the bands on the gel. Lane 11 contains a band at approximately 11 kb which in Figure 3.1 (B) appears as a smear. For the remaining plant species, samples that contained plasmids were collected and represented on a single agarose gel shown in Figure 3.2. This is because many of the gels lacked plasmid bands and some only had - at most - two plasmids.

No plasmids were observed for isolates isolated from rapeseed, while the total number of plasmid containing isolates from dandelion, apple and willowherb were 4, 2 and 5 respectively (Figure 3.2). Close examination of the band patterns revealed 14 different profiles, where one profile, consisting of a single band of approximately 3.7 kb, was observed for 10 of the isolates.

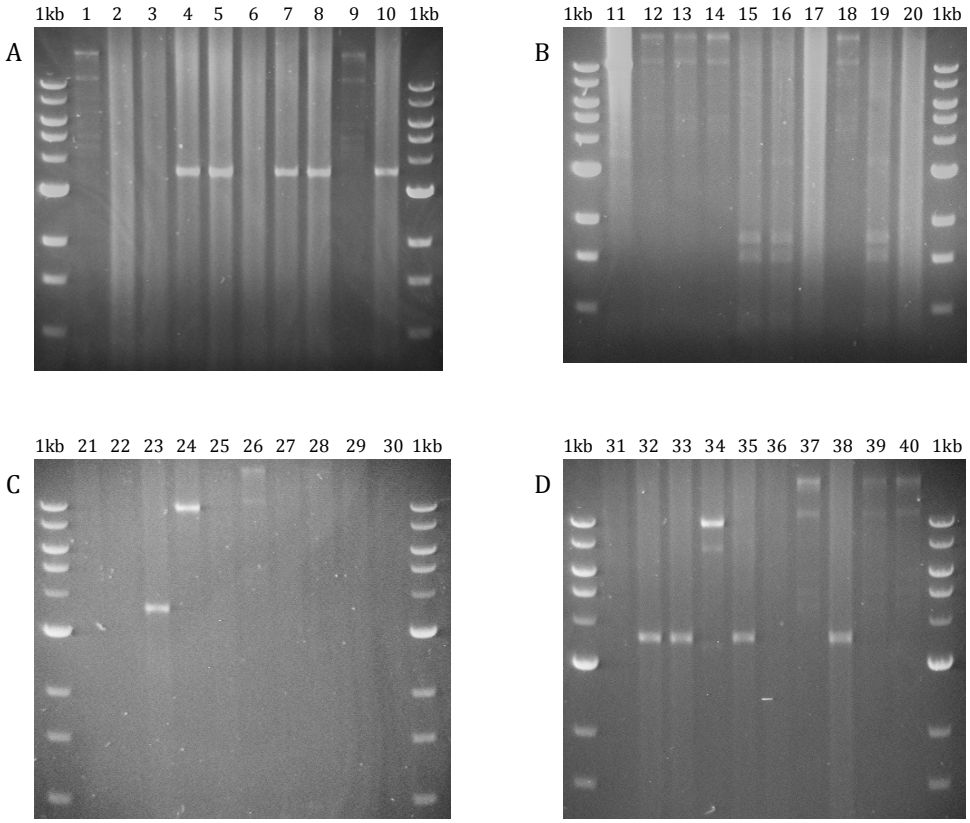

Figure 3.1 Plasmid profiles of bacteria isolated from raspberry. The plasmid profiles of 40 isolates from raspberry are shown from (A) numbers 1-10, (B) numbers 11-20, (C) numbers 21-30, and (D) numbers 31-40.

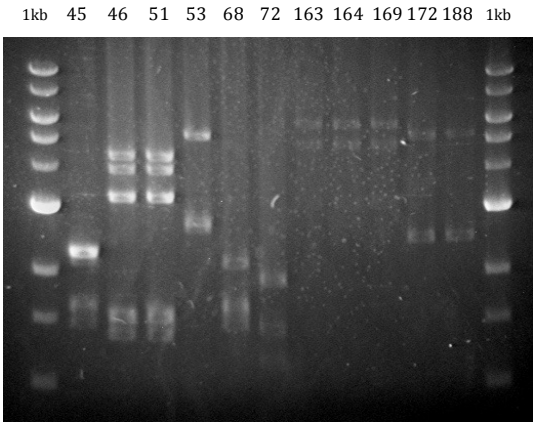

Figure 3.2 Plasmid profiles from isolates gathered from dandelion (numbers 45, 46, 51 and 53), apple (68 and 72), and willowherb (163, 164, 169, 172 and 188).

3.3 Phylogenetic Identification

Approximately 20 isolates from each flower were chosen for further identification by 16S rRNA gene sequencing. Some strains were deliberately chosen to make sure that every plasmid profile was represented. In total, eighty-nine isolates were identified by BLAST analysis of sequences of approximately 1100 bp.

From dandelion, 10 of the isolates were identified as *L. kunkeei* while the other 10 of the isolates, that were grown aerobically, had the closest percent identity to the *Enterobacteriaceae* family. All isolates from rapeseed were identified as *Enterococcus* spp. with high sequence similarities (99 %) to both *Enterococcus haemoperoxidus* and *Enterococcus plantarum.* From apple, 2 of the isolates were identifies as *L. kunkeei* (18 %; 2 of 11 isolates) while 9 isolates were *F. fructosus* (82 %; 9/11). This species was also found in raspberry, which represented 78 % (14/18) while the remaining 22 % were *Lactococcus lactis* (4/18). Samples of willowherb revealed the most diverse bacterial composition, with 6 isolates identified as *L. lactis*, 2 as *Lactobacillus sakei* (10 %; 2/20), 6 as *Lactococcus garvieae* (30 %; 6/20), 5 as *Weissella ceti* (25 %; 5/20) and 1 as *Weissella viridescens* (5 %; 1/20). The distribution of the identified isolates among the different flowers is illustrated in Figure 3.3.

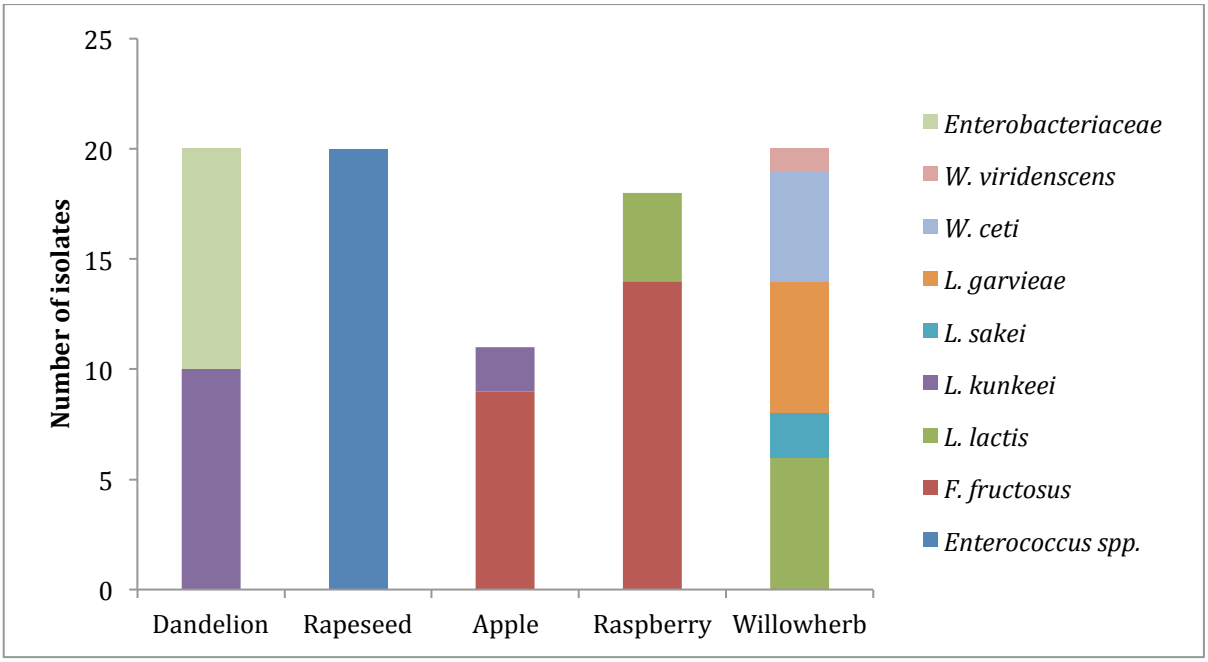

Figure 3.3 Distribution of all identified isolates among various flowers. Isolates from dandelion were identified as *L. kunkeei* and *Enterobacteriaceae*, rapeseed isolates were *Enterococcus* spp., apple isolates were *F. fructosus* and *L. kunkeei*, raspberry isolates were *F. fructosus* and *L. lactis*, and isolates from willowherb were identidied as *L. lactis*, *L. sakei*, *L. garvieae*, *W. ceti* and *W. viridenscens*.

3.4 Sugar Fermentation

Carbohydrate fermentation reactions were recorded for representatives of every species isolated using API50 CHL galleries (Table 3.1). In total, 19 isolates produced acids from 29 of the 49 carbohydrates tested. The results show that all strains except *W. ceti* fermented D-fructose after 1 day, and D-glucose within 7 days. *F. fructosus* strains fermented only 3 sugars within one week, while strains of *L. lactis*, *L. garvieae* and *L. sakei* fermented up to 20 sugars each. Three strains identified as members of the family *Enterobacteriaceae* were also tested for carbohydrate fermentation using the API 50 CHL system, but gave only erratic results that are neither shown nor evaluated more closely.

Table 3.1 Carbohydrate fermentation profile of lactic acid bacteria isolated from flowers.

Carbohydrates[a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16[b]
Glycerol	-	-	-	-	-	-	-	-	i	i	i	-	-	-	-	-
L-arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1d
D-ribose	-	-	-	1d	1d	-	-	-	3d	3d	3d	1d	1d	i	w	1d
D-xylose	-	-	-	1d	1d	-	-	-	-	-	-	-	-	-	-	1d
D-galactose	-	-	-	1d	1d	-	-	-	i	i	i	2d	2d	-	-	1d
D-glucose	+	3d	+	1d	1d	+	+	+	1d	1d	1d	1d	1d	i	1d	1d
D-fructose	1d	1d	1d	1d	1d	1d	1d	1d	1d	1d	1d	1d	1d	-	1d	1d
D-mannose	-	-	-	1d	1d	-	-	-	1d	1d	1d	1d	1d	-	2d	1d
L-rhaminose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	i
D-mannitol	+	+	+	1d	1d	i	+	+	-	-	-	2d	2d	-	-	-
N-acetylglucosamine	-	-	-	1d	1d	-	-	-	1d	1d	1d	1d	1d	i	2d	1d
Amygdallin	-	-	-	2d	3d	-	-	-	i	i	i	1d	1d	-	-	2d
Arbutin	-	-	-	1d	1d	-	-	-	1d	1d	1d	1d	1d	-	-	2d
Esculin ferric citrate	-	-	-	1d	1d	-	-	-	1d	1d	1d	1d	1d	-	1d	1d
Salicin	-	-	-	1d	1d	-	-	-	1d	1d	1d	1d	1d	-	w	2d
D-cellobiose	-	-	-	1d	1d	-	-	-	1d	1d	1d	1d	1d	-	-	1d
D-maltose	-	-	-	1d	1d	-	-	-	+	+	+	2d	2d	i	1d	-
D-lactose	-	-	-	2d	3d	-	-	-	i	i	i	-	-	-	-	2d
D-melibiose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1d
D-saccharose (sucrose)	-	-	-	-	1d	+	+	+	-	-	-	-	-	-	w	1d
D-trehalose	-	-	-	1d	1d	+	+	+	w	w	w	1d	1d	-	1d	1d
Amidon (starch)	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	-	-	-	1d	1d	-	-	-	3d	3d	3d	1d	1d	-	-	2d
D-turanose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-
D-lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	i
D-tagatose	-	-	-	-	-	-	-	-	-	-	-	1d	1d	-	-	-
Potassium gluconate	-	-	-	i	i	i	i	i	-	-	-	w	w	w	i	w

"d" days until positive reaction, "+" positive reaction within 4 to 7 days, "i" intermediate, "w" weak reaction and "-" negative (no color change). [a]Carbohydrates not listed were not fermented by any of the strains. [b]Numbers: 1-3 refer to *F.fructosus* strains B23, B29 and B69; 4-5, *L. lactis* strains B37 and B169; 6-8, *L. kunkeei* strains B45, B53 and B72; 9-11, *Enterococcus* sp. strains B125, B139 and B149; 12-13, *L. garvieae* strains B167 and B187; 14, *W. ceti* strain B177; 15, *W. viridescens* strain B183; 16, *L. sakei* strain B188.

3.5 Cycloheximide

From the cultivation of bacterial strains from apple samples, there were only 17 colony-forming units on the MRS agar plates. In an attempt to yield more isolates, the plates were left at room temperature for aerobic growth for three days. The new colonies

formed were sub-cultured for pure culture isolation and underwent all the same experiments as the other isolates. Plasmid isolation and PCR amplification of the 16S rRNA gene revealed neither plasmids nor amplicons. To address the theory of these isolates not being prokaryote, an experiment with a eukaryote translation inhibitor was conducted (section 2.8).
The results showed no growth in culture tubes containing CHX of concentrations ranging from 5.2 to 156 µg/ml. There was, however, growth in the control tubes containing the bacterium *L. kunkeei*. Microscopy also revealed that the cells were oval/egg-shaped. This strongly suggests that the isolates were yeast.

When grown on MRS agar plates, the yeast produced a sweet-like odor that could resemble the smell of apple cider. This prompted us to investigate if the pigment production and smell were medium dependent. Two isolates denoted 47 and 48 were grown on 4 different growth media (MRS, LA, GM17 and BHI). MRS and LA were the two media that gave off the strongest smell and were therefore analyzed with gas chromatography, along with blank samples of both media to correct for false positives. The analysis was performed by Kari Olsen at NMBU.

Many different alcohols together with some esters were detected; see Appendix 2; Figure A2.1 and A2.2. What affect these compounds could have on the floral nectar, or even if they are produced by the yeast while inhabiting the nectar, is unknown and need more investigation. It could be interesting to see if any of the compounds produced in a significant amount could have any effect on the behavior of the honeybee. A deep literature research and possibly a learning experiment conducted on honeybees would be to recommend.

4. Discussion

LAB were isolated from fresh flowers of five different plant species. The isolates were characterized using plasmid profiling, 16S rDNA sequencing and fermentation profile. Two FLAB species, *L. kunkeei* and *F. fructosus*, were identified in samples from dandelion, apple and raspberry. Rapeseed harbored only *Enterococcus* spp. and willowherb had the most diverse LAB flora consisting of *L. garvieae*, *L. sakei*, *W. ceti* and *W. viridescens.* The LAB were compared with LAB isolated from honeybee gut sampled in parallel with the flowers blooming period. Comparison of the LAB strains and plasmid profiles suggest a positive correlation between the LAB microbial flora in honeybee gut and the flowers within their foraging area.

4.1 Lactic Acid Bacteria Isolated from Flowers

The results showed that the LAB flora of flowers from different plant species varied significantly between some plant species and less between others. Dandelion, apple flower and raspberry harbored the fructophilic LAB species *L. kunkeei* and *F. fructosus*, which have in previous studies also been isolated from flowers, and are known to inhabit fructose rich niches. Rapeseed harbored only *Enterococcus* spp. while willowherb had the most diverse LAB flora consisting of *L. garvieae*, *L. sakei*, *W. ceti* and *W. viridescens.*

L. kunkeei and *F. fructosus* have previously been isolated from several flowers, while *Enterococcus* spp. are readily found in the environment and are commensal members of gut communities in mammals and birds (30). The remaining LAB species, isolated from willowherb, have as far as we know not been isolated from fresh flowers before. These LAB species have for the most part been detected in processed meat, fish or dairy products (31). *L. garvieae* has also been identified in vegetable sprouts (32), and both *L. garvieae* and *W. ceti* have been shown to be fish pathogens (33, 34). However, a relative of *W. ceti, W. confusus*, has recently been isolated from the gut of honeybees (35). Obviously, there is a lot more to learn about the microflora of bees and flowers.

Nectar composition is greatly diverse among different plant species as means to attract different pollinators. Although every plant species collected are of importance for the

honeybee pollinator, indicating a similar nectar composition in general, the nectar compositions may be different enough to cause the different LAB flora observed. For example, nectar from willowherb and rapeseed may not contain enough fructose for FLAB growth. One the other hand, the results may also indicate that the bacterial extraction method used only extracts the most dominant bacterial species. We did not discriminate against different parts of the flower, except for the stem and leaves, which may be considered as a source for the variation of LAB species observed between the plant species, where some LAB may originate from either petals, pollen or nectar.

The fermentation profiles corresponded reasonably well with the literature, and strains of the same specie also showed the same profile, with *L. lactis* as the only exception.
One of the two strains of *L. lactis* fermented sucrose while the other did not. The two strains of *L. lactis* were isolated from different flowers and had different plasmid profiles. This is because the metabolism of sucrose is controlled by plasmid genes and is therefore strain dependent (36).

4.2 Comparing the Results to Lactic Acid Bacteria Isolated from Honeybee Gut

To compare how the LAB isolated from flowers are related to LAB found in honeybee gut, the results from the present study were compared to the results of a parallel MSc thesis conducted by Ingvild Gallefoss (37)(REF), characterizing the gut microbiota of the honeybee, and with similar methods and procedures. Bacterial samples from honeybee gut were provided by the LMG group at the Norwegian University of Life Sciences. Honeybees had been collected from within the hive at the same time points for flower sample collections, and each bacterial sample contained about 10 honeybee guts. All results mentioned about LAB isolated from honeybee gut samples were kindly provided by I. Gallefoss. The flowers collected represent a main pollination target at any given time point.

4.2.1 Comparison of the LAB Species

An overall comparison of the LAB species, regardless of seasonal progression, revealed that 45 % of all isolates from honeybee gut were *L. kunkeei* compared to 15 % of the isolates from flower samples. Both flower samples and honeybee gut samples contained

about 29 % *F. fructosus.* *Bifidobacterium asteroides* accounted for 25 % of all identified LAB strains in honeybee samples, as did *Enterococcus* spp. in flower samples.

With focus on the progressing season and the following change of pollination targets, a comparison of the LAB isolates from flowers and honeybee gut in relation to their time of collection is shown in Figure 4.1. Some correspondence can be seen between the samples collected in middle of May, late May and late June.

In middle of May, the main flowering period of dandelion, *L. kunkeei* was the only species found in both flower and honeybee samples. In late May, the main flowering period for fruit flowers and autumn rapeseed, this species was still dominant in the honeybee gut sample, while *F. fructosus* was dominant in the apple sample.

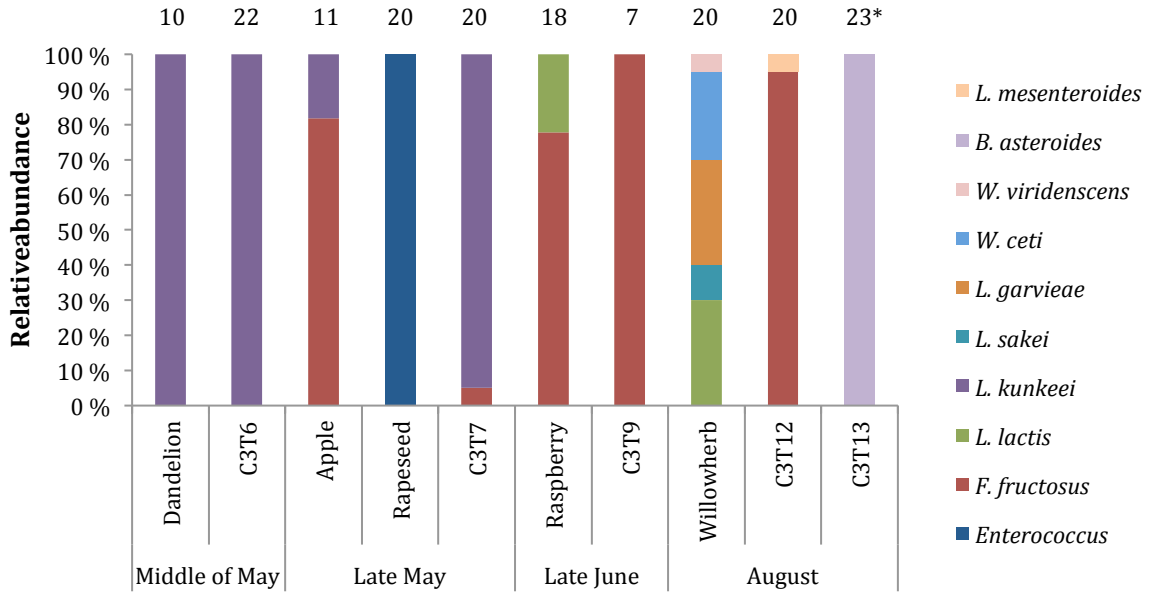

Figure 4.1 The bacterial composition of flower samples compared to samples of the honeybee gut on their respective time points of collection. Data of bacterial strains isolated from honeybee gut samples (C3T6, C3T7, C3T9, C3T12 and C3T13) were kindly provided by Gallefoss 2016 (REF). The relative abundance represents the number of isolates normalized to 100 % of LAB species analyzed. *Number of isolates.

In late June, almost 80 % of the identified isolates from raspberry were *F. fructosus,* while this was the only species identified in the honeybee sample. Seemingly, the LAB composition in the honeybee gut shifts from *L. kunkeei* to *F. fructosus* between middle of May and late June when comparing C3T6, C3T7 and C3T9. One sample from August (C3T12) also contained almost only *F. fructosus*, but this corresponded poorly with the willowherb sample and the other honeybee gut sample (C3T13) collected in August.

Actually, none of the identified bacteria on neither rapeseed nor willowherb were found in any of the samples from honeybee gut. Likewise, no *B. asteroides* were observed in any of the flower samples. *L. lactis* and the other LAB found in flower samples that were absent in the honeybee gut, has as far as we know not been identified in other studies conducted on the honeybee gut.

4.2.2 Comparison of the Plasmid Profiles

In order to evaluate the relatedness between the LAB isolated from flowers and honeybee guts, the plasmid profiles were compared. The plasmid profiles from honeybee gut samples showed greater diversity than the profiles from flowers samples. A total of 36 different profiles distributed among 140 isolates, which accounts for about 74 % of the total number isolated bacteria from honeybee gut, were observed. In contrast, only 21 % of the isolates from flowers contained plasmids with 14 different profiles.

L. kunkeei have previously been shown to comprise many plasmids with high diversity among different strains (38). The same results were shown for the *L. kunkeei* strains isolated from C3T6 and C3T7. Eighteen of the total 34 different profiles were assigned to *L. kunkeei*, and almost every profile was observed for more than one isolate. In flower samples, *L. kunkeei* was identified for 5 different plasmid profiles where no strains contained the same profile. Although the diversity seemed greater for the plasmid profiles of honeybee samples, the abundance of this species were also greater in honeybee samples, which may partly explain the diversity difference.
F. fructosus showed similar diversity in the flower samples to the honeybee gut samples. Seven plasmid profiles were identified as *F. fructosus* in the honeybee samples, while *F. fructosus* strains from flower samples showed 6 different profiles.

Four of the plasmid profiles from flower samples greatly resembled the band pattern of four profiles obtained from the honeybee samples. For three of these profiles, assigned *L. kunkeei*, there was however a discrepancy between the time points of sampling of the isolates that contained the same profile. One profile sampled from apple in May was seen in an isolate from honeybee gut sampled two weeks earlier, and vise versa. A possible explanation for this may be that the blooming period of dandelion and fruit trees, like apple, are around the same time. Individual honeybees, or bees from the same

hive, could therefore have visited both flowers before they got sampled. The majority of the *F. fructosus* strains from both flowers and honeybee gut samples contained a profile consisting of only one band of approximately 3.7 kb, indicating a prevalence of this strain in both flower and honeybee gut samples.

4.3 Bacterial Transmission between Flower and Honeybees

The rapeseed sample was dominated by another flora than the other flowers, and no enterococci were observed in the honeybee samples. Honeybees may prefer apple flowers to rapeseed in this period, or even dandelion, as the bloom period for all three flowers overlap. The fact that the rapeseed field where the flowers were collected from is located more than 4 kilometers from NMBU campus almost certainly means that the honeybees located on campus did not visit these flowers. There is a possibility that there are other rapeseed fields closer to the hive, but these flowers would have to be examined for LAB flora as well before some conclusions can be drawn concerning these sampling results.

Due to the seemingly rapid loss of *L. kunkeei* in the honeybee gut, there are strong indications towards a floral transmission. However, there is no clear evidence for which way the transmission occur. This is in agreement with the hypothesis of several other studies. Ushio et al. (39) demonstrated that the microbial community composition on a flower surface changed after contact with an insect, and they suggested that the microbes are transferred from the insect to the flower although they did not exclude the possibility of the other way around. Aizenberg-Gershtein et al. (40) compared the microbial community in honeybee gut with both covered and uncovered flowers. They found that the microbial flora in the honeybee gut differed more from the covered flower than the uncovered flower, supporting the hypothesis that honeybees may introduce bacteria to the flower.

The honeybees were collected from within the hive, meaning that they did not discriminate against honeybee age and development stage. As the honeybee gut has been shown to vary with age and also gut compartments, any discrepancies in the honeybee samples can also be influenced by these factors. One the other hand, even if the individual honeybee forager tends to stick to one type of flower at a time, bacterial

flora from different flowers can be transferred back to the hive by many different foragers, making the flora more diverse within the hive than on a particular flower. This may contribute to the greater variety of plasmid profiles seen in the honeybee gut samples compared to the flower samples. Possible contamination/transmission from the surrounding environment is quite obvious, but the contamination pressure from the in-hive environment complicates interpretation of the results. The data sets are relatively small to give any hard evidence, but there are many indications toward a positive correlation between the LAB microbial flora in honeybee gut and the flowers within their foraging area.

Further work is advised with a closer examination of the LAB flora from the plant species collected in the study, where parallel samplings possibly could discard bias related to methodological or personal errors. Sampling of honeybees while they visit the flower could perhaps bypass age related influence in contrast to in-hive sampling.

4.4 Conclusion

Flowers provide diverse habitats for microorganisms, and as shown in the present study, the LAB composition in five different plant species varied among them. *L. kunkeei* and *F. fructosus* were the most abundant of all the identified species and is in correlation with other studies on LAB found in flowers. The LAB flora of the honeybee gut seems to shift from *L. kunkeei* to *F. fructosus* through May to late June, and *F. fructosus* was also found as the most abundant LAB in one of the samples collected from honeybee guts in August. This is in accordance with the LAB flora found on dandelion and apple in May and raspberry in late June. These results agrees well with earlier finding of a numerical variation of LAB in the honeybee crop across seasons (5) and a seasonal shift of *L. kunkeei* in the gut of summer bees and winter bees (41) where they suggested that floral transmission could explain the sporadic findings of this specie.

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APPENDIX 1 – Growth Media, Buffers and Tables

Preparation of MRS Media Supplemented with Fructose

A volume of 7.5 ml 40 % fructose was added to 26 g MRS broth. Water was then added to the final volume of 500 ml giving the final concentration of 0.6 % fructose. The solution was distributed on culture tubes in volumes of 6 ml before autoclaving.

Buffers

TE-buffer (Tris EDTA):

1mM Tris-HCl pH 8.0 and 100µM EDTA pH 8.0.

50X TAE stock:

A bottle was filled with approximately 700 ml dH_2O before 242 g Tris-Base, 100 ml 0.5 M EDTA and 57.1 ml Glacial Acetic Acid were added. The flask was filled up to 1 liter with dH_2O and mixed on a magnetic stirrer.

1X TAE-buffer:

100 ml 50X TAE was mixed with 4.9 liters dH_2O.

Tables

Table A1.1 Restriction endonucleases and their restriction sites, incubation- and kill temperature.

Enzyme	Sequence	Incubation temperature	Heat kill
SpeI	A/CTAGT	37 °C	80 °C
XbaI	T/CTAGA	37 °C	65°C
XhoI	C/TCGAG	37 °C	65°C

Table A1.2 Dilution series showing volumes of cycloheximide and the final concentrations.

Culture tube	1	2	3	4	5
5.2 mg/mL CHX working Solution [µL]	0	5	20	50	150
CHX Final Concentration [µg/mL]	0	5.2	20.8	52	156

APPENDIX 2 - Results

Diagrams of GC analysis results

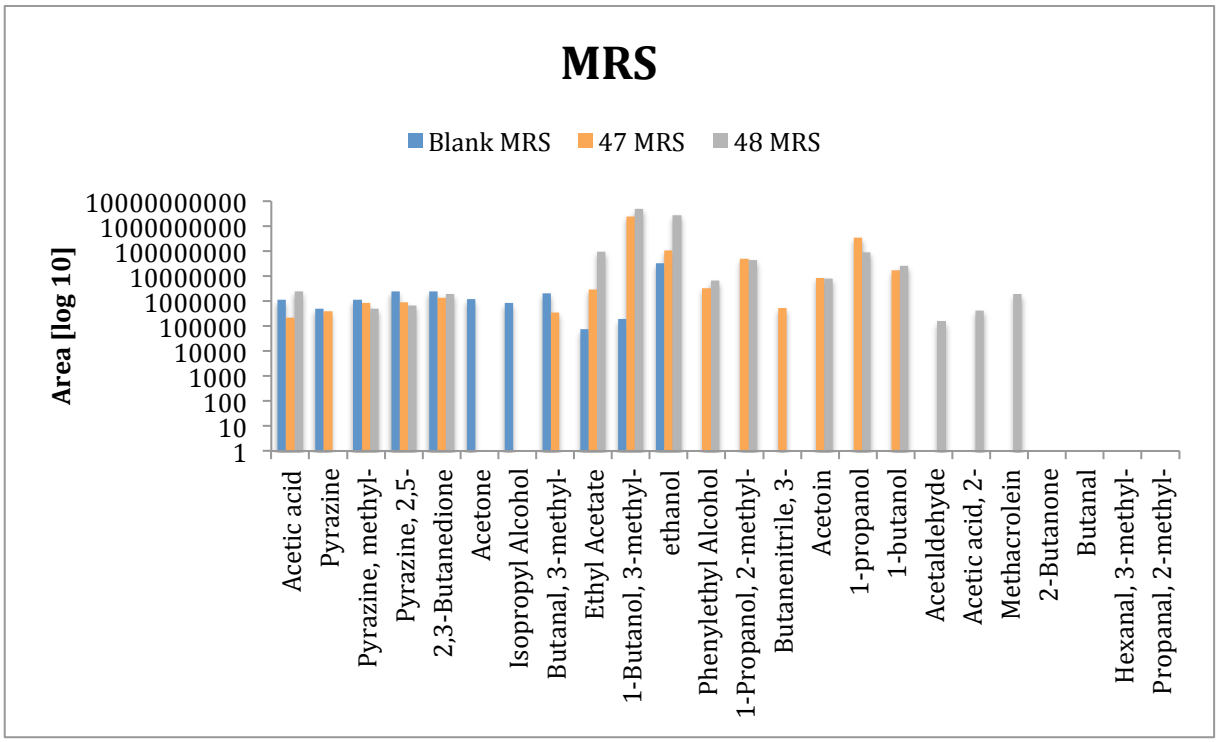


Figure A2. 1. GC analysis results of volatiles produced by two yeast isolates from apple flower grown on MRS medium compared with a blank sample of MRS medium.

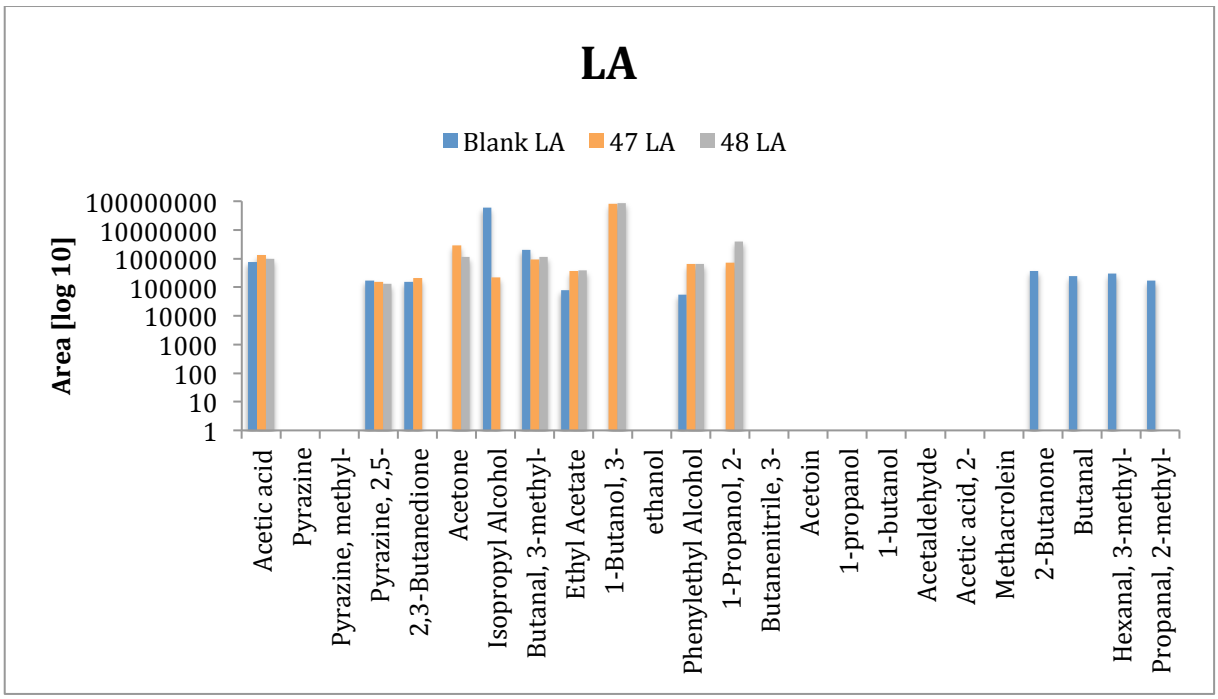


Figure A2. 2. GC analysis results of volatiles produced by two yeast isolates from apple flower grown on LA medium compared with a blank sample of LA medium.

APPENDIX 3 - Protocols

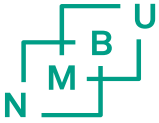
Plasmid Isolation Protocol

1) Inoculate a single colony from a pure culture plate in 6 ml MRS + fructose and incubate at 30 °C overnight.
2) Transfer the ON-culture to a 12 ml falcon tube and centrifuge at 400 rpm for 5 minutes.
3) Decant and discard the culture media.
4) Dissolve the cell pellet in 500 µl TE-buffer by vortex or pipetting and transfer the suspension to a new 1,5 ml micro centrifuge tube.
5) Centrifuge at max speed for 1 minute in a table centrifuge.
6) Decant and discard the supernatant.
7) Make a mix of enzymes by adding 5µl Lysozyme and 5 µl Mutanolysine multiplied by the number of samples.
8) Add 250 µl Solution I to the cell pellet. Vortex to mix thoroughly.
9) Add 10 µl of the enzyme mix and invert the tubes to mix.
10) Place the tube on a water bath with 37 °C for 10 minutes.
11) Add 250 µl Solution II. Invert and gently rotate the tube several times to obtain a clear lysate. A 2-3 minutes incubation time may be necessary.

    NB! Avoid vigorous mixing to avoid dissolving chromosomal DNA and a lower plasmid purity. Do not exceed 5 minutes incubation.
12) Add 350 µl Solution III. Immediately invert several times until a flocculent white precipitate forms.

    NB! It is important that the solution is mixed well and immediately after the addition of Solution III to avoid local precipitation.
13) Centrifuge at maximum speed for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.
14) Insert a HiBind DNA Mini Column into a 2 ml Collection Tube.
15) Transfer the cleared supernatant from Step 13 by carefully aspirating it into the HiBind DNA Mini Column.
16) Centrifuge the column at max speed for 1 minute.
17) Discard the filtrate and reuse the collection tube.
18) Add 500 µl HBC Buffer.
19) Centrifuge at max speed for 1 minute.

20) Discard the filtrate and reuse the collection tube.
21) Add 700 µl DNA Wash Buffer.
22) Centrifuge at max speed for 30 seconds.
23) Discard the filtrate and reuse the collection tube.
24) Repeat step 21 through 23.
25) Centrifuge the empty HiBind DNA Mini Column at max speed for 2 minutes to dry the column.
26) Transfer the HiBind DNA Mini Column to a new 1,5 ml micro centrifuge tube.
27) Add 30 µl Elution Buffer.
28) Let the column sit at RT for 1 minute.
29) Centrifuge at max speed for 1 minute.
30) Repeat step 27 through 29.
31) Store the eluted DNA at -20 °C.



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