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BIOSCIENCE ENGINEERING

# The gut microbiota of bumblebees: a treasure chest of biodiversity and functionality

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## English summary

Bumblebees are efficient generalist pollinators and are important for food production and ecosystem integrity. Commercial bumblebee colonies are used worldwide to enhance the pollination of agricultural crops. Because of the high density and potentially higher pathogen susceptibility of commercial bees, these bees can become reservoirs with high pathogen prevalence of which pathogens can spill back to wild bumblebees. Several studies indicate that the bumblebee gut microbiota contributes to its hosts' health and that it consists of several novel species of bee specific taxa with unknown functionality. The implementation of probiotics may therefore benefit commercial bumblebee health and increase their resistance against pathogens which in turn may reduce pathogen spillover.

The diversity of the bumblebee gut microbiota has mainly been studied by cultivation–independent approaches but these studies lack taxonomic resolution. In addition, the availability of microbial isolates facilitates the analysis of their functional potential. Hence, a first goal was to properly and thoroughly inventorise the cultivable bumblebee gut microbiota through an extensive isolation campaign, coupled to state of the art species-level identification of isolates. Microbial isolates were obtained from the guts of 60 bumblebees belonging to four *Bombus* species by using a rich medium (AC agar) in combination with selective media for the isolation of LAB (MRS agar) and bifidobacteria (MTPY and M144 agar) at 37°C and different atmospheres. By using these isolation conditions and the high-throughput MALDI-TOF MS tool for dereplication, a set of 1940 isolates were obtained belonging to 52 species. We demonstrate that the bacterial species diversity of the bumblebee gut as suggested by 16S rRNA amplicon studies is considerably underestimated as several phylotypes are represented by multiple species and several novel species were isolated for the first time in this thesis. We also show that specific yeast species can be highly prevalent in the bumblebee gut and a proper evaluation of their diversity and functional potential is mandatory. While representatives of most phylotypes reported earlier and detected in the present study were isolated, the isolation of two low abundant but frequently detected phylotypes i.e. Gamma-II and Lacto-4, remained elusive. In accordance with previously reported studies, we found significant differences between the microbial community profiles of different bumblebee species which might be explained by a different host ecology or physiology.

A second goal was to explore the functional potential of the isolates through pathogen inhibition assays and a pectin degradation assay. The parasite *Crithidia bombi* is highly prevalent in bumblebees and is associated with reduced ovary growth and slow colony growth. A 96-well inhibition assay was developed and implemented to test if supernatant of the isolates can inhibit growth of *C. bombi*. Six isolates were obtained with *in vitro* activity against *C. bombi*. The active compound of five *Weissella* isolates is likely hydrogen peroxide while that of a *Streptomyces albidoflavus* isolate remains elusive and is probably non-proteinaceous. Honeybees suffer from severe overwintering declines which are caused by a combination of factors like pesticide use and pathogens. As the honeybee gut microbiota is similar to that of bumblebees, microbial isolates from the bumblebee gut with activity against honeybee pathogens might also be good candidates for honeybee probiotics. Therefore, a well diffusion assay was implemented to investigate if the isolates are able to inhibit growth of the honeybee pathogens *Paenibacillus larvae* and *Melissococcus plutonius* and the bee pathogen *Ascosphaera apis*. Several isolates were obtained with the capacity to inhibit growth of these pathogens *in vitro* but the mechanism of action remains speculative. Pectin is a compound of the pollen wall which is toxic to bees and degradation of pectin by gut micro-organisms may enhance the release of undigested nutrients and reduce pectin-mediated toxicity. Only one isolate was obtained which degraded pectin *in vitro*.

The bumblebee gut microbiota consists of several novel species. A third goal was therefore to formally describe and name these novel species by performing the required polyphasic taxonomic studies and by making reference cultures of these novel species available to the scientific world through their deposit in public culture collections. In the present thesis we described novel species belonging to the genera *Bombella*, *Gilliamella*, *Lactobacillus*, *Weissella*, *Convivina*, *Bifidobacterium* and *Apibacter*. Of these novel species *Bombella intestini*, four novel *Gilliamella* species, *Lactobacillus bombicola*, *Bifidobacterium commune* and *Apibacter mensalis* belong to bacterial phylotypes previously detected in the bumblebee gut.

## Nederlandstalige samenvatting

Hommels zijn efficiënte bestuivers van verschillende plantenfamilies en zijn belangrijk voor de voedselproductie en het behoud van ecosysteem integriteit. Commerciële hommelkolonies worden wereldwijd ingezet om de bestuiving van landbouwgewassen te bevorderen. Door de hoge densiteit aan commerciële kolonies en de mogelijks hogere vatbaarheid van commerciële hommels kunnen deze hommels een reservoir vormen met een hoge pathogeen prevalentie waarvan pathogenen kunnen overgedragen worden naar wilde hommels. Verschillende studies geven aan dat de darmmicrobiota van hommels bijdraagt tot hun gezondheid en dat deze microbiota bestaat uit verschillende nieuwe soorten van bij-specifieke taxa met een ongekende functionaliteit. Het gebruik van probiotica zou de resistentie van commerciële hommels tegen pathogenen kunnen verhogen wat op zijn beurt de overdracht van pathogenen kan verminderen.

De diversiteit van de darmmicrobiota van hommels werd voornamelijk bestudeerd door middel van cultivatie-onafhankelijke benaderingen waarvan de taxonomische resolutie beperkt is. Daarenboven vergemakkelijkt de beschikbaarheid van microbiële isolaten het onderzoek naar hun functionele potentieel. Een eerste doel van deze thesis was daarom om een grondig inventaris te maken van de kweekbare micro-organismen in de hommeldarm door middel van een uitgebreide isolatiecampagne gekoppeld aan state of the art identificatie van de isolaten op soortniveau. Microbiële isolaten werden verkregen uit de darmen van 60 hommels behorende tot vier hommelsoorten door het gebruik van een rijk medium (AC agar) in combinatie met selectieve media voor de isolatie van melkzuurbacteriën (MRS agar) en bifidobacteriën (MTPY en M144 agar) bij 37°C en verschillende atmosferen. Door het gebruik van deze isolatiecondities en de MALDI-TOF MS dereplicatie methode met een hoge doorvoercapaciteit werd een set van 1940 isolaten bekomen die 52 soorten omvatten. We tonen aan dat de bacteriële soortendiversiteit van de hommeldarm zoals gesuggereerd door 16S rRNA amplicon studies aanzienlijk onderschat is aangezien meerdere soorten verkregen werden voor verschillende fylotypes en verschillende nieuwe soorten voor het eerst gedetecteerd werden in deze thesis. We tonen ook aan dat specifieke gistsoorten behoorlijk prevalent kunnen zijn in de hommeldarm en dat een grondige evaluatie van hun diversiteit en functionaliteit noodzakelijk is. Hoewel representatieve isolaten verkregen werden voor de meeste eerder gerapporteerde fylotypes en fylotypes gedetecteerd in deze studie, konden geen isolaten verkregen worden voor twee laag-abundante fylotypes i.e. Gamma-II en Lacto-4 die frequent gedetecteerd worden. In overeenstemming met eerder gerapporteerde studies werden

in deze thesis ook significante verschillen gevonden tussen de microbiële gemeenschapsprofielen van verschillende hommelsoorten wat mogelijks verklaard kan worden door een verschillende ecologie en fysiologie.

Een tweede doel van deze thesis was om het functionele potentieel na te gaan van de isolaten via pathogeeninhibitie testen en een pectine afbraak test. De parasiet *Crithidia bombi* is zeer prevalent in hommels en is geassocieerd met gereduceerde groei van de eierstokken en een vertraagde koloniegroei. Een 96-well inhibitie test werd ontwikkeld en uitgevoerd om na te gaan of de isolaten de groei van *C. bombi in vitro* kunnen voorkomen. Zes isolaten werden verkregen met activiteit tegen *C. bombi*. De actieve component van vijf *Weissella* isolaten is waarschijnlijk waterstofperoxide terwijl dat van een *Streptomyces albidoflavus* isolaat ongekend blijft en waarschijnlijk geen eiwit is. Honingbijen leiden onder enorme sterftes bij het overwinteren die veroorzaakt worden door een combinatie van factoren zoals het gebruik van pesticiden en ziekte veroorzaakt door pathogenen. De darmmicrobiota van honingbijen is gelijkaardig aan die van hommels en microbiële isolaten uit de hommeldarm met activiteit tegen honingbij pathogenen zouden ook geschikte honingbij probiotica kunnen zijn. Een well diffusie test werd uitgevoerd om na te gaan of de isolaten de groei van de honingbij pathogenen *Paenibacillus larvae* en *Melissococcus plutonius* en bijenpathogeen *Ascosphaera apis* kunnen tegengaan. Verschillende isolaten werden verkregen met de capaciteit om de groei van deze pathogenen tegen te gaan *in vitro* maar het werkingsmechanisme blijft speculatief. Pectine is een component van de pollenwand die toxisch is voor bijen en de afbraak van pectine door micro-organismen in de darm zou de vrijstelling van onverteerde nutriënten kunnen bevorderen en pectine gerelateerde toxiciteit verminderen. Slechts één isolaat werd verkregen dat in staat is om pectine af te breken *in vitro*.

De darmmicrobiota van hommels bestaat uit verschillende nieuwe soorten. Een derde doel was daarom om deze nieuwe soorten formeel te beschrijven en te benoemen door het uitvoeren van de vereiste polyfasische taxonomische studies en door referentie culturen beschikbaar te maken voor de wetenschappelijke wereld door ze te deponeren in publieke cultuurcollecties. In deze thesis werden nieuwe soorten beschreven die behoren tot de genera *Bombella*, *Gilliamella*, *Lactobacillus*, *Weissella*, *Convivina*, *Bifidobacterium* en *Apibacter*. *Bombella intestini*, vier nieuwe *Gilliamella* soorten, *Lactobacillus bombicola*, *Bifidobacterium commune* en *Apibacter mensalis* behoren tot fylotypes die reeds eerder gedetecteerd werden in de hommeldarm.

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# List of Abbreviations

## A

| | |
|---|---|
| AAB | acetic acid bacteria |
| AC | all culture agar |
| AES | agri-environmental schemes |
| ANI | average nucleotide identity |
| ANOSIM | Analysis Of Similarity |

## B

| | |
|---|---|
| bp | base pairs |

## C

| | |
|---|---|
| COI | cytochrome oxidase I mitochondrial gene |
| CTAB | hexadecyltrimethylammonium bromide |

## D

| | |
|---|---|
| DMSO | dimethylsulfoxide |
| DDH | DNA-DNA hybridisation |
| DPD | Digital Protologue Database |

## E

| | |
|---|---|
| EM | enrichment medium |
| EPS | extracellular polymeric substances |

## F

| | |
|---|---|
| FAME | whole-cell fatty acid methyl esters |

## G

| | |
|---|---|
| GBDP | Genome Blast Distance Phylogeny |

## H

| | |
|---|---|
| HPLC | high pressure liquid chromatography |
| HSP | high-scoring segment pair |

## I

| | |
|---|---|
| ITS | internal transcribed spacer |

## J

| | |
|---|---|
| JGI | Joint Genome Institute |

## L

| | |
|---|---|
| LAB | lactic acid bacteria |
| LB | Luria-Bertani |

## M

| | |
|---|---|
| MALDI-TOF MS | matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry |
| MIDI | Microbial Identification System, Sherlock version 3.10 |
| MRM | microbial resource management |
| MRS | De Man Rogosa Sharpe |
| MTPY | modified tryptone phytone yeast extract |

## N

| | |
|---|---|
| NMDS | non-metric multidimensional scaling |
| nt | nucleotides |

## O

| | |
|---|---|
| OGRI | overall genome relatedness indices |
| OTU | operational taxonomic unit |

## P

| | |
|---|---|
| PCR | polymerase chain reaction |
| PGA | polygalacturonic acid |

## Q

| | |
|---|---|
| QUAST | Quality Assessment Tool for genome assemblies |

## R

| | |
|---|---|
| RAPD | random amplified polymorphic DNA analysis |

## S

| | |
|---|---|
| SCFA | short-chain fatty acids |
| SIMPER | similarity percentage |
| STEP | Status and Trends for European Pollinators |

## T

| | |
|---|---|
| TRFLP | terminal restriction fragment length polymorphism |
| TSB | tryptic soy broth |

# Part I

# Introduction:
# The microbiology of bumblebees

CHAPTER 1

# Bumblebees

Bumblebees are important pollinators of tomatoes, sweet pepper and many other commercial crops and wild plants. There is currently great concern about their worldwide decline and that of other pollinators like butterflies and hoverflies which may have a detrimental economic impact and may create an instable ecosystem [Grixti et al., 2009; Potts et al., 2010; Biesmeijer et al., 2006]. These declines are presumably caused by different factors that include climate change, change in agricultural practices, pesticide and insecticide use and pathogen spillover [Colla et al., 2006; Meeus et al., 2011; Cariveau et al., 2014; Fitzpatrick et al., 2007]. In the present chapter, bumblebees will be introduced and their lifecycle, food, intestinal tract and immune system will be discussed. Subsequently, the importance of bumblebees as pollinators and causes of bumblebee population declines will be outlined.

## 1.1 Bumblebee taxonomy

The *Apinae* subfamily of bees includes 19 tribes of which the *Bombini*, *Euglossini*, *Apini* and *Meliponini* are corbiculate bees (Figure 1.1) [Schultz et al., 2009]. Corbiculae or pollen baskets are flat, shiny areas on the outside surface of the bee hindleg surrounded by spiky hairs and are used to transport collected pollen. The *Euglossini* are solitary bees while members of the other corbiculate *Apinae* are all eusocial. Eusociality is characterized by a non-reproductive worker caste that is morphologically different from the queen, by a division of labour and by overlapping generations [Goulson, 2003]. Bumblebees (*Bombus* sp.) are often considered primitively eusocial because the workers are, although smaller, not morphologically different from the queen and reproduction by workers is common [Sadd et al., 2015].

Bumblebees and honeybees (*Apis* sp.) are the best known bees mainly because of their extensive commercial use and because they are excellent pollinators. Bumblebees are also attractive because of their buzzing activity during daylight and their large bodies with bright colours and fuzzy long hairs which make them a pleasure to watch. Bumblebees include approximately 250 species worldwide and prefer cool and flower-rich habitats which can be found in the northern hemisphere. However, a few native species also occur in South America [Goulson, 2003]. In Belgium, approximately 30 bumblebee species occur of which *Bombus terrestris*, *Bombus lucorum*, *Bombus lapidarius* and *Bombus pascuorum* are the most common [Rasmont et al., 2005]. Different bumblebee species differ in several characteristics like tongue length and hence plant preferences, start and duration of the life cycle, habitat preference and manner of feeding their larvae. Although species of most subgenera can be distinguished by their characteristic colour pattern, some bumblebee species have a very similar colour pattern and should be identified by sequence analysis of the cytochrome oxidase I (COI) mitochondrial gene [Carolan et al., 2012].

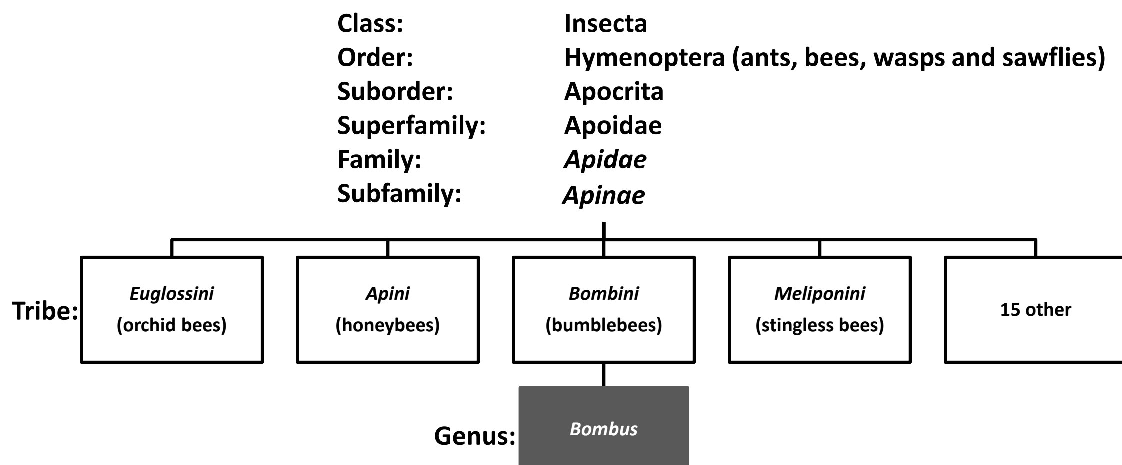


**Figure 1.1:** Taxonomic neighbourhood of the genus *Bombus*.

## 1.2 Bumblebee life cycle

While honeybee colonies are perennial and a queen overwinters with her workers and a honey store, bumblebees have an annual life cycle (Figure 1.2). At the end of the season only the daughter queens (gynes) survive and hibernate [Goulson, 2003]. In spring, bumblebee queens emerge from their hibernation site which often is a hole in the ground. The timing of emergence depends on the bumblebee species with e.g. *B. terrestris, B. lucorum* and *B. hypnorum* queens already emerging in February or March and *B. pascuorum* and *B. lapidarius* queens emerging in late March or April [Prys-Jones and Corbet, 2011]. Once a queen emerges, she starts feeding and looking for a nesting site.

Preferred nesting sites also depend on the bumblebee species and can be abandoned holes of rodents, cavities in trees and walls of houses and dense vegetation on the surface of the ground. The queen builds the nest with nearby materials such as moss and grass and collects pollen and nectar. The pollen is arranged into a pollen lump within which 8 to 16 eggs are laid and the nectar is stored into a wax pot on which the queen feeds while incubating the brood. Within four days the eggs hatch and the larvae start feeding on the pollen. After approximately 10 to 14 days of larval development including four larval phases or instars, the larvae start spinning a silk cocoon and pupate. Two weeks later the pupae bite their way out of the cocoon and newly emerged workers appear which are white. After 24 hours they develop their characteristic colour patterns. From this moment on, the queen can stay in the nest to lay eggs which will be taken care of by workers. Older bumblebees will start collecting food (pollen and nectar), which is called foraging, to support growth of the nest.

Compared to honeybee nests which comprise approximately 50000 individuals, bumblebee nests are considerably smaller and hold 50 to 400 individuals [Goulson, 2003; Prys-Jones and Corbet, 2011]. Once a nest contains enough workers, the queen switches to rearing male bumblebees (drones) and daughter-queens. After a few days males leave the nest, never to return. They will forage and spread a pheromone to attract queens for mating. Most bumblebee species mate only once. The mated queens will build up large fat reserves to be able to hibernate. Finally, the old queen and the rest of the nest will die [Goulson, 2003].

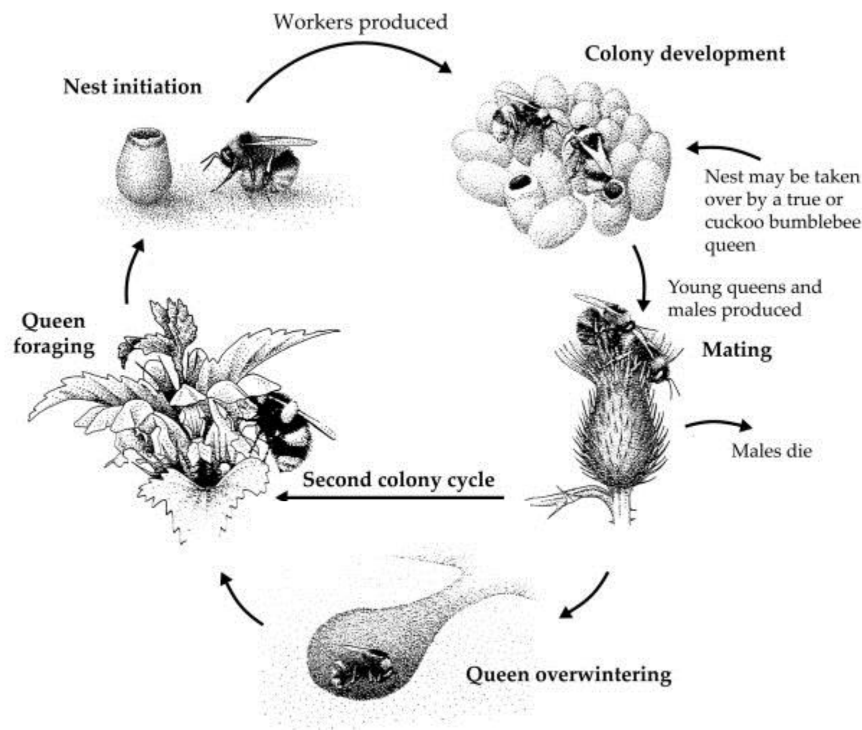


**Figure 1.2:** The bumblebee life cycle. Figure from Prys-Jones and Corbet [2011].

## 1.3 Bumblebee food

All bees are phytophagous and feed on pollen and nectar [Goulson, 2003; Prys-Jones and Corbet, 2011]. Nectar consists mainly of glucose, fructose and sucrose and is an excellent energy source. In addition, collecting nectar is necessary for bumblebees to obtain water. In contrast to honeybees, bumblebees rarely collect water during foraging and need to extract it from nectar. Bees collect nectar by protruding their proboscis (tongue) into the corolla of a flower. Therefore, the flower species that a bumblebee is able to access when foraging for nectar largely depends on the length of its proboscis. Short-tongued species (e.g. *B. terrestris, B. pascuorum* and *B. lapidarius*) collect nectar from rather short, open flowers or bite holes in the corolla of deeper flowers (a practice referred to as nectar robbing). The nectar reward varies between plant species and even between flowers of the same plant and bumblebees switch to other plant species after a succession of low nectar rewards of their preferred flower species. In addition to the variability of the nectar concentration and composition, nectar of some flowers (e.g. *Tilia*, *Rhododendron* and *Salix*) can also contain sugars which are toxic to bees like e.g. mannose [Goulson, 2003; Prys-Jones and Corbet, 2011; Pawlikowski, 2010]. Mannose cannot be digested by bees and accumulates in their cells causing finally bee paralysis [Pawlikowski, 2010]. Although sugars are the main compounds of nectar, it also consists of other nutrients like lipids and proteins.

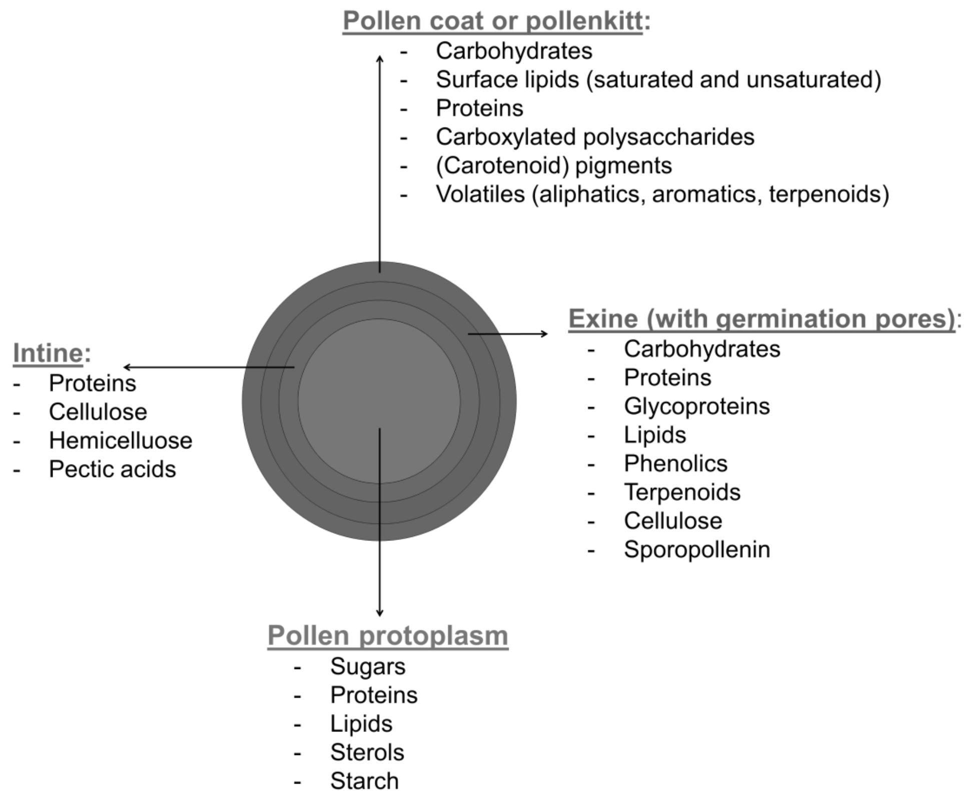


**Figure 1.3:** Schematic overview of the pollen structure with the different layers, pollen coat, exine, intine and protoplasm with their components. Figure from Billiet [2016].

Proteins are necessary for colony development and growth and are especially obtained from the nutrient-rich pollen [Goulson, 2003; Keller et al., 2005; Roulston and Cane, 2000]. Pollen (Figure 1.3) consists of an outer layer (the exine) composed of a poorly characterised and difficult to degrade structure called sporopollenin [Roulston and Cane, 2000]. The exine is covered by the pollenkitt which consists of lipids, proteins and sugars. The inner layer (the intine) consists mainly of pectic acids and cellulose and covers the protoplasm which contains proteins, amino acids, sugars, starch, lipids, minerals and vitamins. The nutritional quality of pollen varies between plant species. Although bumblebees are polylectic (i.e. they collect pollen from different plant families), plants of the *Fabaceae* family (e.g. peas and clovers) are preferred foraging plants [Goulson et al., 2005]. *Fabaceae* pollen contains more proteins and essential amino acids compared to plants of other families [Goulson, 2003; Roulston and Cane, 2000].

## 1.4 Bumblebee intestinal tract

The hymenopteran intestinal tract comprises three main regions: the foregut, the midgut and the hindgut (Figure 1.4) [Engel and Moran, 2013; Swingle, 1927]. The foregut starts with a pharynx and oesophagus and ends with a honey stomach or crop in which the collected nectar is stored during foraging. The honey stomach continues into the proventriculus which is a small tube forming a valve-like structure together with the oesophageal valve preventing reflux of food from the midgut into the foregut. Just like the hindgut, the foregut is ectodermal and is lined with an exoskeleton which is called the intima. It consists of chitin and cuticular glycoproteins and separates the lumen from the epidermal cell-layer.

The actual stomach of bees is the midgut or ventriculus. The epidermal cells at the anterior end of the midgut secrete a semi-permeable peritrophic membrane which consists of chitin, proteins and carbohydrates and extends through the entire midgut. The peritrophic membrane functions as a barrier to protect the epithelial cells from mechanical and chemical damage. The midgut epithelial cells excrete digestive enzymes and the nutrients obtained through digestion are absorbed.

The hindgut consists of ileum, rectum and anus. The ileum is separated from the midgut by the pyloric valve which is posterior to the Malphigian tubes. These tubes are excretory organs shedding the uric waste from the body cavity into the ileum. Water resorption takes place in the ileum before the food and uric waste is transferred to the rectum for excretion through the anus.

The bee gut atmosphere includes both aerobic and anaerobic regions [Engel and Moran, 2013; Swingle, 1927]. The pH of the honey stomach and hindgut is low (+/- 5) while that of the midgut is neutral (+/- 6.5) [Anderson et al., 2011; Harz, 2015]. The temperature of the abdomen varies from 17.5°C to 39.5°C and mainly depends on active heat production in the thorax by shivering [Stabentheiner et al., 2010].

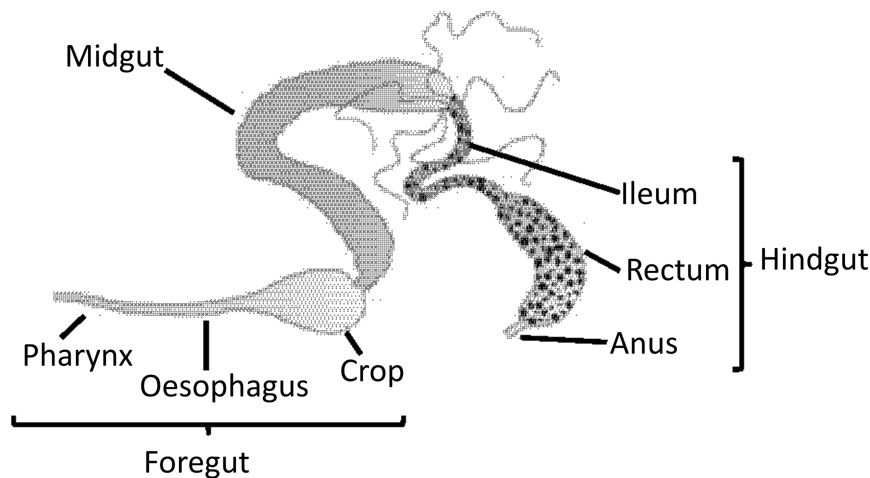


**Figure 1.4:** The intestinal tract of members of the Hymenoptera. Figure adapted from Engel and Moran [2013].

## 1.5 Bumblebee defense system

The bumblebee defense system is composed of colony related and individual related strategies. On the level of the colony, defense against disease is mediated by nest hygiene (grooming and elimination of diseased brood, dead bees and waste) while individual bees are protected by a constitutive and an inducible immune system [Engel and Moran, 2013; Evans et al., 2006; Sadd et al., 2015]. The constitutive protection is mediated by the gut barrier, the low pH of the honey stomach, the melanisation of pathogens by the phenoloxidase pathway and phagocytosis by the haemocytes. Strikingly, bumblebees are also capable of avoiding pathogen contaminated flowers [Fouks and Lattorff, 2011].

In addition, bumblebees have an inducible immune system with pathogen recognition proteins and several immune pathways (Toll, Imd, JAK/STAT and JNK) mediating the production of antimicrobial proteins (e.g. defensins, lysozyme, peptidoglycanase) and reactive oxygen species. The immune system of bumblebees also confers increased protection upon secondary exposure to a pathogen after several weeks. The bumblebee gene repertoire related to immunity and detoxification of xenobiotics is similar to that of honeybees but is relatively small (+/- 150 genes) compared to that of flies and mosquitos which have three times as many related genes [Engel and Moran, 2013; Evans et al., 2006; Sadd et al., 2015].

## 1.6 The importance of bumblebees for pollination

Bees collect nectar and pollen from flowers and while doing so, they transfer pollen from one flower to the next which aids the fertilization and seed setting of plants. Bees pollinate approximately two thirds of the world's agricultural crops with a commercial value of hundreds of billions of dollars annually [Goulson, 2003; Losey and Vaughan, 2006; Hatfield et al., 2012]. Eighty-five percent of the world's flowering plants depends on animal pollination of which the majority is bee pollination. Of the 250 crop species in Europe, approximately 150 species are insect-pollinated. Thus, the ecological and commercial impact of bees on the world is enormous [Goulson, 2003; Losey and Vaughan, 2006].

Bumblebees are very efficient pollinators. In contrast to honeybees, bumblebees also forage on cold and rainy days. Their hairy bodies are useful to transfer pollen from one flower to the next. In addition, bumblebees forage faster than honeybees and perform 'buzz pollination' which is necessary for the pollination of *Solanaceae* plants like tomatoes and sweet pepper. Bumblebees grasp the anthers of these flowers and shake the pollen out through the rapid vibration of their flight muscles which makes a buzzing sound. The beneficial effects of bumblebees on food production are widely appreciated by farmers which buy commercial nests to pollinate their crops (e.g tomatoes, sweet pepper and courgette) in greenhouses [Goulson, 2003; Klatt et al., 2014]. As bumblebees are generalist pollinators (Table 1.1) that pollinate a large diversity of plant species, the disappearance of bumblebee species or decline of bumblebee populations may affect multiple plant populations and ecosystem integrity [Biesmeijer et al., 2006; Goulson, 2003].

**Table 1.1:** Crops known to benefit from bumblebee pollination. Table from Goulson [2003].

| Crop | | Need for pollination | Other probable pollinators |
|---|---|---|---|
| ***Actinidiaceae*** | | | |
| ***Actinidia deliciosa*** | Kiwifruit | *** | H |
| ***Brassicaceae*** | | | |
| ***Brassica napus*** | Rape | * | H,S |
| ***Brassica campestris*** | Turnip rape | ** | H,S |
| ***Asteraceae*** | | | |
| ***Helianthus annuus*** | Sunflower | *** | H,S |
| ***Ericaceae*** | | | |
| ***Vaccinium macrocarpon*** | Cranberry | *** | H |
| ***Vaccinium angustifolium*** | Lowbush blueberry | *** | H |
| ***Vaccinium ashei*** | Rabbiteye blueberry | *** | H |
| ***Vaccinium corymbosum*** | Highbush blueberry | *** | H |
| ***Grossularidaceae*** | | | |
| ***Ribes grossularia*** | Gooseberry | * | H |
| ***Ribes* spp.** | Curants | ** | H |
| ***Malvaceae*** | | | |
| ***Gossypium* spp.** | Cotton | * | H,S |
| ***Fabaceae*** | | | |
| ***Phaseolus multifloris*** | Runner bean | ** | H |
| ***Phaseolus lunatus*** | Lima bean | * | H |
| ***Vicia faba*** | Field or broad bean | ** | H,S |
| ***Vicia villosa*** | Vetch | ** | H,S |
| ***Medicago sativa*** | Lucerne or alfalfa | *** | H,S |
| ***Melilotus* spp.** | Sweet clover | *** | H,S |
| ***Trifolium* spp.** | Clovers | *** | H,S |
| ***Glycine max*** | Soya bean | * | H |
| ***Lupinus* spp.** | Lupins | ** | - |
| ***Rosaceae*** | | | |
| ***Prunus avium*** | Sweet cherry | *** | H |
| ***Prunus cerasus*** | Sour cherry | *** | H |
| ***Prunus communis*** | Pear | *** | H |
| ***Prunus domestica*** | Plum | ** | H |
| ***Prunus malus*** | Apple | *** | H,S |
| ***Rubus fruticosus*** | Blackberry | ** | H,S |
| ***Rubus ideaus*** | Raspberry | * | H |
| ***Rutaceae*** | | | |
| ***Citrus* spp.** | Orange, lemon, etc. | * | H,S |
| ***Solanaceae*** | | | |
| ***Solanum melongena*** | Aubergine | * | H |
| ***Lycopersicon esculentum*** | Tomato | * | H,S |
| ***Capsicum* spp.** | Pepper | * | H |
| ***Curcubitaceae*** | | | |
| ***Cucumis melo*** | Muskmelon | ** | H |
| ***Cucumis sativus*** | Cucumber | *** | H |
| ***Citrullus lanatus*** | Watermelon | *** | H |
| ***Cucurbita* spp.** | Squash, pumpkin, gourd | *** | H,S |

H, honeybees; S, solitary bees. ***Insect pollination essential and *insect pollination improves yield to some degree.

## 1.7 Bumblebee population declines

Like many other insect pollinators, bumblebee populations are declining worldwide both in species richness and local geographic range extent with most evidence available from North America and Europe [Biesmeijer et al., 2006; Cameron et al., 2011; Colla and Packer, 2008; Fitzpatrick et al., 2007; Kosior et al., 2007; Berezin and Beiko, 2002; Potts et al., 2010; Rasmont et al., 2005]. In Europe, 13 bumblebee species became extinct between 1950 and 2000 in at least one country of the 11 assessed ones and four *Bombus* species (*B. armeniacus*, *B. cullumanus*, *B. serrisquama* and *B. sidemii*) became extinct in all of these countries [Kosior et al., 2007]. Of the 68 species, 31 (46%) are regressing which indicates that the situation in Europe is severe (Figure 1.5) [Kosior et al., 2007]. More specifically, in Belgium four bumblebee species (*B. confusus*, *B. cullumanus*, *B. distinguendus* and *B. pomorum*) have not been reported after 1990 and are assumed to be extinct [Kosior et al., 2007; Rasmont et al., 2005]. Rasmont *et al.* showed that the population decline of bumblebees in Belgium can be explained partially by a drop in the presence of legume crops, which have deep corollas, as long-tongued bee species declined relatively more than short-tongued species [Rasmont et al., 2005]. However, factors causing bumblebee population declines differ worldwide [Biesmeijer et al., 2006; Cameron et al., 2011; Fitzpatrick et al., 2007] and declines are probably the result of a synergistic effect of increasing pressure from anthropogenic factors and natural stressors like pathogens and parasites [Kosior et al., 2007].

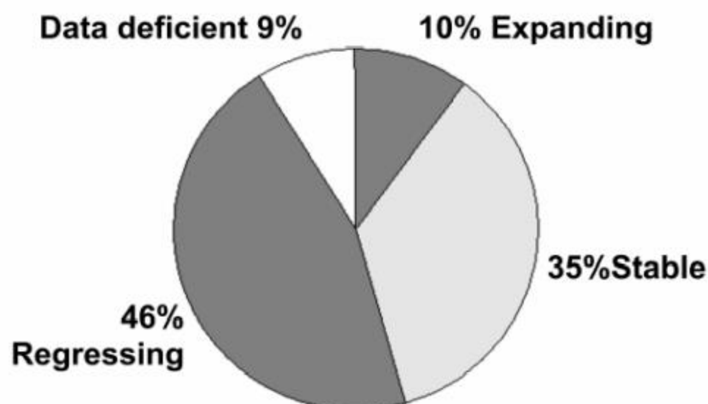


**Figure 1.5:** Summary of the regression status of European bumblebees. Figure adapted from the IUCN Bumblebee Specialist Group report 2013 [Williams and Jepsen, 2013].

## 1.8 Causes of bumblebee population declines

### 1.8.1 Anthropogenic factors

A main cause of pollinator population declines is undoubtedly an intensified agriculture. Especially in the second half of the twentieth century, agriculture intensified and led to a massive reduction of unfarmed land and an increase of monocultures [Goulson, 2003; Fitzpatrick et al., 2007]. To increase production, farmers started to produce silage instead of hay. The production of silage accelerates and increases harvest but requires earlier and more frequent mowing which leads to less wild flowers and less unimproved land for nesting. The introduction of fertilizers made the practice of crop rotation unnecessary. An important group of plants used for crop rotations are the *Fabaceae* (e.g clover) which are preferred foraging plants for long-tongued bumblebees and the reduced availability of these plants is associated with the loss of these bees [Carvell et al., 2006; Fitzpatrick et al., 2007; Goulson, 2003]. Due to intensified land use, large flowerless landscapes nowadays exist which are food deserts for bees. Bumblebees are highly susceptible to a reduction in food availability as they do not store a lot of food and forage needs to be available throughout a colony's lifecycle. Populations of some bumblebee species (e.g. *B. terrestris*, *B. pascuorum* and *B. lapidarius*), however, seem to be stable or increasing and not surprisingly, these are species with rather broad diets [Goulson et al., 2005, 2008a].

Habitat fragmentation results in smaller bumblebee populations which are separated over large distances which in turn provokes inbreeding depression. This genetic inbreeding reduces bumblebee fitness and influences the infection success of pathogens and parasites [Goulson, 2003; Whitehorn et al., 2014].

Another factor is the potential contamination of flowers by pesticides. Often used pesticides are juvenile hormone analogues and neonicotinoids (e.g. imidacloprid) and the sublethal effects of doses used in agriculture have been described repeatedly [Feltham et al., 2014; Kessler et al., 2015; Lundin et al., 2015; Mommaerts et al., 2009, 2006].

### 1.8.2 Pathogen emergence

The most commonly reported bumblebee pathogens are the protozoa *Crithidia bombi, Nosema bombi* and *Apicystis bombi.* The trypanosome *C. bombi* (Figure 1.6) is the most prevalent bumblebee pathogen with up to 50% of spring queens infected [Goulson, 2003; Shykoff and Schmid-Hempel, 1991]. Due to rapid transmission within and between colonies almost all colonies are infected by the end of the season and up to 67% of the individuals is infected by multiple strains [Salathe and Schmid-Hempel, 2011; Shykoff and Schmid-Hempel, 1991]. It was first assumed that *C. bombi* is transmitted through nectar [Cisarovsky and Schmid-Hempel, 2014]. However, *C. bombi* was not detected in field nectar and its survival significantly decreased in high sugar concentrations (>0.2 g/ml). Therefore, this pathogen is more likely transmitted through physical contact with flower surfaces [Cisarovsky and

Schmid-Hempel, 2014]. In the bumblebee gut, *C. bombi* occurs in two different morphologies, the amastigote which has no flagella and the flagellated promastigote which is able to adhere to the gut wall [Schmid-Hempel and Tognazzo, 2010; Wheeler et al., 2013]. In the gut, this parasite benefits from the available nutrients and multiplies by clonal and sexual reproduction after which novel infective cells are shed in the faeces [Cisarovsky and Schmid-Hempel, 2013; Erler et al., 2012]. The virulence of the pathogen is condition-dependent, causing no mortality without stress. Mortality increases by 50% when workers are starved [Brown et al., 2000]. Infection is also associated with reduced ovary growth, slow colony growth and reduced colony founding [Shykoff and Schmid-hempel, 1991; Yourth et al., 2008]. Bumblebees will elicit an immune response upon infection with *C. bombi* mainly consisting of released antimicrobial proteins (e.g. defensin and abaecin) [Deshwal and Mallon, 2014].

The prevalence of the neogregarine pathogen *A. bombi* is considered low [Maharramov et al., 2013; Plischuk et al., 2011]. This pathogen infects and disrupts fat tissue which has severe effects on hibernating queens, and which reduces colony founding success. The microsporidian pathogen *N. bombi* has a variable prevalence [Huth-Schwarz et al., 2012; Shykoff and Schmid-Hempel, 1991] and is obligately intracellular, infecting multiple body tissues like Malpighian tubes, thorax muscles and fat body. This parasite reduces worker survival and fitness [Otti and Schmid-Hempel, 2007]. Another *Nosema* species, *N. ceranae* which was long thought to be a specific pathogen of honeybees is now often detected in bumblebees as well [Gamboa et al., 2015; Graystock et al., 2014]. Moreover, several pathogens can infect both honeybees and bumblebees (e.g. *Ascosphaera apis* and *Spiroplasma melliferum*) and are presumed to be transmitted between the two (e.g. Deformed Wing Virus and Acute Bee Paralysis Virus) [Goulson, 2003; Maxfield-Taylor et al., 2015; Meeus et al., 2010; Parmentier et al., 2016]. Two economically important and widely distributed bacterial pathogens of honeybee brood are *Paenibacillus larvae* and *Melissococcus plutonius* [De Graaf et al., 2006; Forsgren, 2010]. These pathogens cause American and European foulbrood disease, respectively, which are devastating diseases characterized by a foul smell. *A. apis* causes chalkbrood disease in honeybee larvae and both vegetative and reproductive stages of the fungus have been detected in adult bumblebees [Maxfield-Taylor et al., 2015; Vojvodic et al., 2012; Aronstein and Murray, 2010]. Some other *Ascosphaera* species are pathogenic and have been detected in the gut of solitary bee larvae (*Megachile* spp., *Osmia* spp. and *Nomia* spp.) [Ravoet et al., 2014; James and Skinner, 2005]. The prevalence of the foulbrood and chalkbrood disease pathogens ranges between one and six percent in honeybee apiaries and varies between geographic regions and over time [Budge et al., 2014; Garrido-Bailon et al., 2013; Chemurot et al., 2016; Forsgren, 2010].

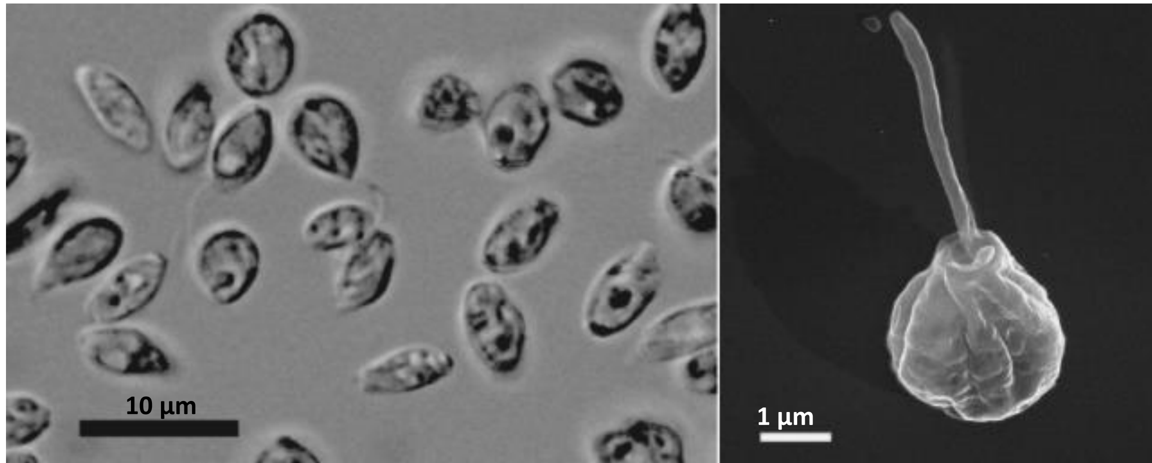


**Figure 1.6:** Differential interference contrast microscopy (left) and scanning electron microscopy image (right) of *C. bombi*. Figure adapted from Schmid-Hempel and Tognazzo [2010].

### 1.8.3 Commercial bumblebee nests

Commercial bumblebee nests are often used for the pollination of greenhouse crops such as tomatoes and sweet pepper. However, Whittington et al. [2004] showed that up to 73% of the pollen collected by commercial bumblebees came from outside the greenhouse. Bees often escape from greenhouses and are able to influence native populations in multiple ways. Introduced species can weaken or displace native populations through competition for food and nesting sites, introgression (i.e. gene flow from one species into the gene pool of another) and spillover of pathogens and non-native pathogens. Pathogen spillover is the spread of pathogens from a highly infected host population into a sympatric host population [Colla et al., 2006]. Spillover can operate in both directions because both native and commercial bees can act as a pathogen reservoir. Colla et al. [2006] and Graystock et al. [2014] observed a higher prevalence of *C. bombi* and *A. bombi* in wild bumblebees near greenhouses containing commercial bumblebees (Figure 1.7) and associations have been observed between the use of commercial bumblebees and local declines of wild bumblebees [Colla et al., 2006; Graystock et al., 2014; Schmid-Hempel et al., 2013; Goulson and Hughes, 2015; Graystock et al., 2016a]. Although the implementation of hygienic measures and pathogen screening in rearing facilities can prevent the export of infected colonies, commercial bumblebees can become infected with pathogens carried by wild bees at the site of use. Because of the high density of commercial bumblebees in greenhouses, these bees can act as a reservoir with high pathogen prevalence of which pathogens can then spill back to wild bees [Graystock et al., 2014].

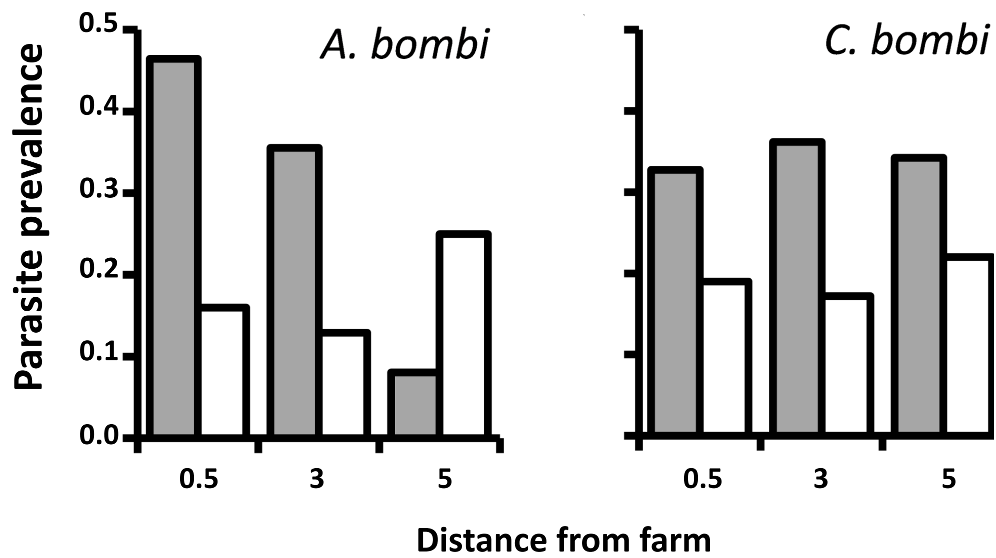


**Figure 1.7: The effect of commercially produced bumblebees on parasite prevalence in wild bumblebees.** The proportion of bumblebees sampled which were positive for *A. bombi* and *C. bombi*. Bumblebees were sampled at 0.5, 3 or 5 km from greenhouses that were either using (grey columns) or not using (white columns) commercially produced bumblebee colonies. Figure from Graystock et al. [2014].

# CHAPTER 2

## The bumblebee gut microbiota

The importance of the gut microbiota to human health is nowadays widely appreciated. Functions associated with the human gut microbiota are digestion of food, detoxification of xenobiotics, production of essential nutrients like vitamins and protection against pathogens through colonisation resistance, production of antimicrobial compounds and control of the gut barrier function [Jandhyala et al., 2015]. Also in insects similar beneficial and even essential associations with gut bacteria have been reported [Engel and Moran, 2013]. The gut microbiota of insects consists of bacteria, archaea, viruses, fungi and protists. However, in insects most gut micro–organisms are bacteria and studies therefore often focus on the latter. In the present chapter a brief overview is given of different types of insect-gut communities and methods used to study microbial diversity. Subsequently, this chapter will elaborate on the gut microbiota composition of bumblebees and the known bumblebee-gut microbiota interactions. Finally, the potential use of pro- and prebiotics in commercial bumblebee rearing facilities will be discussed.

## 2.1 The insect gut microbiota

Insects are by far the most diverse animals and consist of almost one million species which occupy a variety of niches [Engel and Moran, 2013]. The composition, species richness and functionality of the insect gut microbiota are highly diverse. Therefore, this section will address a representative selection of insect gut communities (Table 2.1).

### 2.1.1 Composition and localisation

The insect gut microbiota composition consists mainly of Proteobacteria and Firmicutes and is shaped by different characteristics of the host like gut morphology, habitat, food preferences and social behaviour [Engel and Moran, 2013]. The insect gut consists of a foregut, midgut and hindgut. The foregut and hindgut are protected from direct contact with micro-organisms through a cuticle layer lining the epithelial cells and the midgut sheds a peritrophic matrix which divides the midgut into an endo- and ectoperitrophic area. Micro-organisms are present in the endoperitrophic area and the matrix obstructs their exposure to the midgut epithelium. In some insects' fore- and hindgut, microbial communities are established by the formation of biofilms [Vasquez et al., 2012; Vilanova et al., 2016] and the predominant localisation of micro-organisms in insect guts is often associated with specialised structures [Dillon and Dillon, 2004]. In the plataspid bug *Megacopta punctatissima* symbiotic bacteria colonise midgut invaginations called caecae or crypts [Fukatsu and Hosokawa, 2002]. In termites, most symbiotic micro-organisms are present in the paunch, which is an enlarged compartment of the hindgut [Warnecke et al., 2007]. Compartmentalisation of the gut is associated with larger microbial communities (e.g. termites) and insects with small gut communities (e.g. fruit flies and aphids) typically have long, narrow guts [Engel and Moran, 2013].

### 2.1.2 Acquisition

Micro–organisms can be acquired from the environment and food or can be transmitted through social contact (Table 2.1). Transmission fidelity allows gut microbiota-host interactions to become stable and the establishment of specialised interactions is facilitated in social insects like termites, honeybees and bumblebees. Gut micro–organisms can be transmitted in these insects through feeding of the larvae and contact with faeces of nest mates. However, transmission can also be guaranteed in non-social insects. *Ishikawaella capsulatus* is a specialised gut symbiont of *M. punctatissima* which is essential for host development and is transmitted efficiently by defecation of ovipositioning females [Fukatsu and Hosokawa, 2002]. Females deposit small, brown capsules filled with symbiont cells under the eggs and these capsules are eaten by the nymphs after emergence. Subsequently, *I. capsulatus* colonises crypts in the posterior part of the midgut. The prominent symptoms of symbiont-free nymphs are retarded development and abnormal body colouration [Fukatsu and Hosokawa, 2002].

Environmental micro–organisms can also be acquired reliably every generation. The bean bug *Riptortus pedestris* acquires a specific *Burkholderia* symbiont from soil and lack of acquisition of this symbiont negatively affects host development [Kikuchi et al., 2005, 2007].

Although the gut microbiota composition is uniform among individuals of some insect species, in most insects this composition is variable and seems to depend largely on the habitat and food. However, environmentally acquired bacteria can be very important to insects as micro-organisms associated with insect food are often capable of manipulating the food and help the insect host with food digestion [Engel and Moran, 2013].

### 2.1.3 Functionality

Micro–organisms associated with the gut of insects are responsible for a myriad of functions ranging from food digestion and nutrient provisioning to protection against chemical and physical burdens and influencing the behaviour of the host (Table 2.1).

*Rhodococcus rhodnii* provides B vitamins to its host (*Rhodnius prolixus*) which stimulates larval development [Eichler and Schaub, 2002]. Upon infection of *R. prolixus* with the trypanosome *Trypanosoma rangeli* the concentration of the symbiont decreases which leads to retarded larval development and increased larval mortality [Eichler and Schaub, 2002].
Insects which feed on restricted diets like plant sap and wood (e.g. termites) depend on their gut symbionts to obtain sufficient essential vitamins and amino acids and to digest complex compounds like lignocellulose. Termites are wood-feeders and can be divided in lower and higher species [Ohkuma, 2008; Warnecke et al., 2007]. The lower termites only feed on wood and interact with flagellated protists. The protists produce cellulases in the gut which together with host enzymes enable the complete digestion of cellulose [Ohkuma, 2008]. Higher termites have a wider feeding habit and lack protists for the digestion of cellulose. In these insects, Spirochaetes and *Fibrobacter* species and host enzymes are responsible for the degradation of cellulose and xylan [Warnecke et al., 2007]. The bacterial community in termite guts is species-rich and no cultivated representatives are available for more than 90% of the detected phylotypes (i.e. environmental DNA sequences or groups of sequences typically sharing more than 97% 16S rRNA gene sequence similarity). At least three novel candidate phyla have been detected in termites and are referred to as TG1, TG2 and TG3 [Ohkuma, 2008]. Both in lower and higher termites, $H_2$ metabolism, $CO_2$ reductive acetogenesis and atmospheric $N_2$ fixation are important microbial processes in the gut [Ohkuma, 2008; Warnecke et al., 2007]. In lower termites, protists degrade cellulose and produce $H_2$ and $CO_2$ as waste products which are used by the acetogenic bacteria. These bacteria produce acetate which is the main energy source of termites. In higher termites similar processes are performed by Spirochaetes and *Fibrobacter* species. Wood is depleted in nitrogen and termites obtain amino acids by $N_2$-fixing bacteria in the gut [Ohkuma, 2008; Warnecke et al., 2007].

Gut symbionts can also protect their hosts against toxins like plant defensive compounds and insecticides. The *Burkholderia* symbiont of *R. pedestris* degrades the insecticide fenitrothion and evokes host resistance [Kikuchi et al., 2012]. In *Drosophila* the gut microbiota fine-tunes the immune system and protects the host against pathogen infections [Broderick and Lemaitre, 2012].

Another interesting example of enhanced protection of the host by its gut microbiota is the interaction of the reed beetle with *Macropleicola* species [Kolsch et al., 2009]. The reed beetle pupates in a mud layer under water and is protected by a rigid cocoon which also allows the availability of oxygen. *Macropleicola appendiculatae* and *Macropleicola muticae* colonise the intestine and Malpighian tubes of this beetle and secrete the building material for the rigid cocoon.

Bacteria also influence the mating preferences of *Drosophila melanogaster* by altering its sex pheromone [Broderick and Lemaitre, 2012] and in the grasshopper *Schistocerca gregaria* gut bacteria produce the major compounds of the aggregation pheromone (phenol and guaiacol) which maintains the aggregation of this insect in groups [Dillon et al., 2002].

Apart from these beneficial interactions, interactions of insects with gut bacteria can also be harmful like infection of pea aphids with the pathogen *Pseudomonas syringae* [Stavrinides et al., 2009]. Another example is production of volatiles by *Staphylococcus sciuri*, an inhabitant of the pea aphid gut, which attract pea aphid predators [Leroy et al., 2011].

The above mentioned interactions all comprise symbionts that live in the host's gut. However, several insect interactions with intracellular bacteria are known. These bacteria can be obligate like *Buchnera aphidicola* in aphids or facultative like *Wolbachia pipientis*, *Spiroplasma* species and *Hamiltonella defensa* which are not essential to the host and can occasionally be transmitted horizontally [Engel and Moran, 2013]. All aphids contain *B. aphidicola* in specialised cells called bacteriocytes and obtain essential nutrients from their *Buchnera* symbionts. The genome of *B. aphidicola* is extremely reduced but genes for the production of essential amino acids and vitamins are maintained [Engel and Moran, 2013].

Although insect guts are mostly dominated by bacteria, yeasts can also be present and have been associated with a variety of functions like nutrient provisioning, food digestion, protection against pathogens and plant toxin degradation [Gonzalez, 2014]. Yeasts are rich sources of vitamins, amino acids and trace metals and insect larvae can depend on yeast nutrition during development [Becher et al., 2012]. The guts of honeybees, bumblebees, stingless bees and solitary bees mainly comprise members of the yeast genera *Candida*, *Cryptococcus*, *Metschnikowia* and *Starmerella* [Rosa et al., 2003]. While most of the yeast species in the bee gut are found in bee related habitats, some yeasts like *Candida etchelsii* and *Zygosaccharomyces* species have also been found in other niches like sausages and lemon juice [Rosa et al., 2003].

**Table 2.1:** Transmission modes, composition and proposed functions of representative insect gut communities. Table adapted from Engel and Moran [2013].

| Insect host species | Transmission route | No. of major spp. | Examplar taxa | Consistency among hosts | Host food | Proposed roles in hosts |
|---|---|---|---|---|---|---|
| Plataspid bug: *Megacopta punctatissima* | Maternal: egg capsule | 1 | *Ishikawaella capsulatus* (Proteobacterium) | Uniform | Plant sap | Development and growth |
| Alydid bug: *Riptortus pedestris* | Environment | 1<br>1 | *Burkholderia* sp. (Proteobacterium) | Uniform | Plant sap | Nutritional?, degradation of insecticides |
| Reed beetle: *Macroplea* sp. | Maternal egg-smearing | 1<br>1 | *Macropleicola* spp. (Proteobacterium) | Uniform | Plant cells | Production of cocoon material |
| *Rhodnius prolixus* | Maternal egg-smearing | 1? | *Rhodococcus rhodnii* (Actinobacterium) | Uniform | Blood | Nutrient provisioning |
| Honeybees and bumblebees: *Apis* spp. *Bombus* spp. | Social transmission | 6–9 | *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* spp. | Uniform | Pollen and nectar | Digestion, protection against parasites, other? |
| Lower termite: *Reticulitermes speratus* | Social transmission | >300 | Flagellates, Bacteroidetes, Spirochaetes Proteobacteria, Firmicutes | Uniform | Dry wood | Nutrient provisioning, nitrogen recycling, fixation, lignocellulose digestion, fermentation |
| Higher termite: *Nasutitermes* sp. | Social transmission | >300 | Spirochaetes, Fibrobacteres, Bacteroidetes, Firmicutes, Acidobacteria, Proteobacteria, TG3 | Uniform | Detritus | Nutrient provisioning, nitrogen recycling, fixation, cellulose digestion, fermentation |
| Grasshopper: *Schistocerca gregaria* | Acquisition from food | <12 | *Enterococcus*, *Serratia*, *Klebsiella*, *Acinetobacter* | Variable | Plant leaves | Produce compounds of aggregation pheromone |
| Fruit fly: *Drosophila melanogaster* | Acquisition from food | <8 | *Lactobacillus* spp., *Acetobacteraceae*, *Orbaceae* | Variable | Decaying fruit | Prime immune system, affect metabolism and mating preferences |
| Gypsy moth caterpillar: *Lymantria dispar* | Acquisition from food | <8 | *Pseudomonas*, *Enterobacter*, *Pantoea*, *Serratia*, *Staphylococcus*, *Bacillus* | Variable | Plant leaves | Unknown, may increase susceptibility to toxin by affecting midgut epithelial permeability |
| Pea aphid: *Acyrthosiphon pisum* | Environment | Few in healthy aphids | *Staphylococcus*, *Pseudomonas*, *Acinetobacter*, *Pantoea* | Variable | Phloem sap | Mostly pathogenic, produce signalling compounds that attract aphid predators |

## 2.2 Study of microbial diversity: cultivation dependent and independent approaches

The microbial diversity in a particular niche or sample comprises its entire community of bacterial, fungal, protozoal and viral micro-organisms. The species concepts of these groups are abstract definitions and are primarily practical tools to enable the classification and identification of micro-organisms and efficient communication. A bacterial species consists of a monophyletic group of strains which share more than 98.65% 16S rRNA gene sequence similarity and are genetically (>70% DNA:DNA hybridisation) and phenotypically similar [Kim et al., 2014; Vandamme and Peeters, 2014]. With the availability of bacterial genome sequences, the laborious DNA:DNA hybridization approach to determine the degree of genomic similarity has become outdated and has been replaced by measures based on the whole genome sequence (e.g. >96% average nucleotide identity (ANI)) [Vandamme and Peeters, 2014; Richter and Rosselló-Móra, 2009]. A frequently used phylogenetic marker in bacterial diversity studies is the 16S rRNA gene [Clarridge, 2004]. This gene is approximately 1500 base pairs (bp) long, is universally present in bacteria, is slowly evolving because of its essential function in translation and has an extremely low recombination frequency. The 16S rRNA gene sequence includes nine variable regions separated by conserved regions which form candidate sites for primer binding [Clarridge, 2004].

### 2.2.1 Cultivation independent approaches

The microbial diversity of the honeybee and bumblebee gut has been studied primarily by cultivation independent methods [Cox-Foster et al., 2007; Koch et al., 2013; Koch and Schmid-Hempel, 2011b; Martinson et al., 2011, 2012; Meeus et al., 2013, 2015]. Although approaches like terminal restriction fragment length polymorphism (TRFLP) and molecular cloning of 16S rRNA gene sequences are still used, next-generation sequencing (i.e. 16S rRNA amplicon sequencing) has become the gold standard to study microbial diversity. Of the different technologies available, Illumina is a frequently used one and Illumina MiSeq now allows paired-end sequencing of 2 x 300 bp of the variable V3 and V4 regions of the 16S rRNA gene to obtain an amplicon of approximately 460 bp [Schirmer et al., 2015].

The obtained sequence reads are commonly clustered into operational taxonomic units based on a cut-off of 97% 16S rRNA gene sequence similarity. This cut-off is based on the previously used taxonomic cut-off of 97% (now 98.65% [Kim et al., 2014]) for species delineation which was determined for full length 16S rRNA gene sequences. It must also be noted that although bacteria which share less than 98.65% 16S rRNA gene sequence similarity consistently represent different species, bacteria which share more than 98.65% sequence similarity of their entire 16S rRNA gene sequence do not necessarily belong to the same species [Mysara et al., 2016]. Due to the short length of the reads, the resolution of 16S rRNA amplicon sequencing is commonly limited to that of the genus or even family level. The resolution will also depend on the sequenced variable region as the mutation rate

differs over the variable regions for different families [Clarridge, 2004; Mysara et al., 2016].

Theoretically, all the 16S rRNA gene sequences present in a particular sample should be detectable by 16S rRNA amplicon sequencing. However, the obtained sequencing data vary with many factors like the DNA-extraction protocol, the sequencing technology, rRNA operon copy number, the primers and polymerase used and the targeted region of the 16S rRNA gene [Lagier et al., 2015; Gohl et al., 2016; Tremblay et al., 2015; Gihring et al., 2012; Hiergeist et al., 2015]. More effort is needed to lyse Gram positive cells compared to Gram negative ones and an inefficient DNA-extraction can lead to the underestimation of the microbial diversity [Kennedy et al., 2014]. The detection limit of amplicon sequencing also depends on the sequencing depth and low abundant taxa can be missed [Hiergeist et al., 2015]. Simultaneously, the diversity can also be overestimated due to cross-contamination and issues inherent to sequencing technology and polymerase chain reaction (PCR) [Gohl et al., 2016; Tremblay et al., 2015]. The majority of errors observed in Illumina data are substitution errors which can result from problems with enzyme kinetics and inadequate flushing of flow cells [Schirmer et al., 2015]. In the PCR reaction an incomplete 16S rRNA gene sequence can function as a primer which can result in the formation of sequences consisting of fragments from different organisms (i.e. chimeras). These chimeras will multiply during subsequent PCR cycles and can erroneously be interpreted as unique operational taxonomic units [Mysara et al., 2016].

### 2.2.2 Cultivation dependent approaches

A large-scale microbial diversity study performed by Lagier et al. [2015] which combined 16S rRNA amplicon sequencing and cultivation concluded not only that there is an overlap of the taxa detected by means of both approaches but also that both approaches allow the detection of additional taxa. Therefore, a combination of both approaches is appropriate to obtain a more complete image of the microbial diversity of a sample [Lagier et al., 2015; Hugon et al., 2013]. In addition, the availability of microbial isolates allows a more accurate identification through sequence analysis of the whole 16S rRNA gene, of housekeeping genes with a higher phylogenetic resolution or even of the whole genome. It also enables the development of *in vitro* and *in vivo* experiments to explore their functional potential. A cultivation approach also allows the determination of intraspecies variation. Strains of a single species can differ in up to 30% of their gene content which may concern potentially interesting strain-specific traits [Konstantinidis and Tiedje, 2005].

Although cultivation dependent approaches are time-consuming and laborious, recent improvements allow the high-throughput isolation and identification of thousands of isolates from dozens of different cultivation conditions. This approach has been referred to as microbial culturomics [Lagier et al., 2015]. This so-called culturomics is made feasible by the use of colony pickers and time-effective isolate identification through matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS allows the rapid identification of isolates by generating a protein profile for each isolate

which is compared with entries in a validated database [Ghyselinck et al., 2011]. Even if an isolate cannot be identified because, for instance, a reference profile is absent in the database, the tool can be used to group isolates of the same species, a process referred to as dereplication. After dereplication, unknown isolates can be identified by sequencing the 16S rRNA or housekeeping genes of representative isolates from each group [Ghyselinck et al., 2011].

The success of cultivation depends on providing micro–organisms with suitable chemical (nutrients) and physical (pH, atmosphere, light and temperature) growth conditions. The required nutrients can be divided into macro- and micronutrients. Macronutrients are nutrients which are required in large amounts (i.e. carbon, nitrogen, hydrogen, phosphorus, oxygen, sulfur, potassium, magnesium, calcium and sodium) while only trace amounts of micronutrients are needed. Micronutrients include trace elements (e.g. $Fe^{3+}$ and $Mn^{2+}$) and growth factors (vitamins, amino acids, purines and pyrimidines). Growth factors are organic compounds needed in small amounts by micro–organisms not able to synthesize them. Culture media can either be general, allowing the growth of a variety of micro–organisms, or (semi-)selective, allowing the growth of specific micro–organisms.

## 2.3 The bumblebee gut microbiota composition

As mentioned above, the microbial diversity of the honeybee and bumblebee gut has mostly been studied by DNA based approaches like clone libraries and 16S rRNA amplicon sequencing [Koch et al., 2013; Koch and Schmid-Hempel, 2011b; Martinson et al., 2012; Meeus et al., 2015; Mohr and Tebbe, 2006; Moran et al., 2012]. Although different research groups have focused on the isolation and description of specific microbial taxa from the honeybee and bumblebee gut, no truly large-scale isolation campaigns have been reported [Killer et al., 2010b; Kwong and Moran, 2013, 2016b; Olofsson and Vásquez, 2008].

When the bee gut is mentioned in this thesis, we are referring to the combination of honey crop, midgut and hindgut.

The gut microbiota composition of adult honeybees and bumblebees is similar and specific and is dominated by up to five taxa which are consistently present and which are generally not found in other environments. This suggests that these bacteria are adapted to the honeybee and bumblebee gut and likely influence their health. The honeybee and bumblebee gut microbiota is often divided into a core and a noncore community [Kwong and Moran, 2016a; Meeus et al., 2015]. The core is defined as a group of bacteria whose members occur in most of the individual bees, independent of geographic location and which are typically not found in the environment while noncore members have a more erratic occurrence in the gut [Cariveau et al., 2014; Meeus et al., 2015]. The core bacteria of the honeybee and bumblebee gut microbiota are a Gammaproteobacterium (referred to as Gamma-1), a Betaproteobacterium (referred to as Beta) and a Firmicutes (referred to as Firm-5) phylotype. The core of honeybees also consists of a second Firmicutes (referred to as Firm-4) and an

Actinobacterium (referred to as Bifido-1) phylotype which also occur in bumblebee gut communities albeit more sporadically. In addition, the core of bumblebees also consists of a specific Actinobacterium phylotype (referred to as Bifido-3). For a long time the lack of cultivated representatives of each of these phylotypes prevented both a more accurate identification of these organisms as well as an assessment of the taxonomic structure of each of these phylotypes. Other more erratically observed phylotypes in the honeybee or bumblebee gut are listed in Table 2.2 [Kwong and Moran, 2016a; Meeus et al., 2015]. This table presents an overview of the bacterial phylotypes often detected in the honeybee and bumblebee gut and the cultivated bacterial species that belong to each of these phylotypes. Cultivated representatives are available for most of the phylotypes, however, most of them have been isolated from the honeybee gut.

**Table 2.2:** Bacterial phylotypes in the gut of bumblebees and honeybees and the cultivated or candidate species assigned to these phylotypes.
The names of the phylotypes are those used by Meeus et al. [2015] unless stated otherwise. If the phylotype belongs to the core gut microbiota, the host name is indicated in bold character type.

| Phylotype (closest relative) | Cultivated or named species | Host |
|---|---|---|
| Gamma-1 | | **H,B** |
| | *Gilliamella apicola* [Kwong and Moran, 2013] | H,B |
| | Candidatus *Schmidhempelia bombi* [Martinson et al., 2014] | B |
| Gamma-2 | *Frischella perrara* [Engel et al., 2013] | H |
| Gamma-II[a] | | |
| (*Pseudomonas* sp. 91%)[b] | / | H,B |
| Gamma-P | | |
| (*Pseudomonas* spp. 100%)[c] | / | B |
| Gamma-E1 | | |
| (*Buttiauxella agrestis* 100%)[c] | / | B |
| Gamma-E2 | | |
| (*Hafnia alvei* 100%)[c] | / | H,B |
| Gamma[d] | | |
| (*Serratia* sp.)[e] | / | B |
| Beta | *Snodgrassella alvi* [Kwong and Moran, 2013] | **H,B** |
| Alpha-1 | *Bartonella apis* [Kesnerova et al., 2016] | H |
| Alpha-2.1 | | |
| (*Gluconobacter* sp.)[e] | / | H |
| Alpha-2.2 | | H,B |
| | *Parasaccharibacter apium* [Corby-Harris et al., 2014] | H |
| Firm-5/Lacto-1 | | **H,B** |
| | *Lactobacillus kullabergensis*, *L.melliventris*, | H |
| | *L. kimbladii*, *L. helsingborgensis* [Olofsson et al., 2014] | H |
| | *L. apis* [Killer et al., 2013] | H |
| Firm-4/Lacto-2 | | **H**,B |
| | *Lactobacillus bombi* [Killer et al., 2014] | B |
| | *L. mellis* [Olofsson et al., 2014] | H |
| Bin4[f] | *Lactobacillus mellifer* [Olofsson et al., 2014] | H |
| Lacto-3 | | H,B |
| | *Lactobacillus kunkeei* [Endo and Salminen, 2013], | H |
| | *L. apinorum* [Olofsson et al., 2014] | H |
| Lacto-4 | | |
| (*Lactobacillus* sp. 89%)[b] | / | H,B |
| Lacto-5 | | |
| (*Lactobacillus kimchicus* 100%)[c] | / | H,B |

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Continued from previous page.

| Phylotype (closest relative) | Cultivated or named species | Host |
|---|---|---|
| Fructobacillus[b] | *Fructobacillus fructosus* [Endo and Salminen, 2013] | H,B |
| Firm-S | | |
| (*Lactococcus* spp. 100%)[c] | / | B |
| Firm-E | | |
| (*Enterococcus* spp. 100%)[c] | / | B |
| Bifido-1 | | **H**,B |
| | *Bifidobacterium actinocoloniiforme* [Killer et al., 2011] | B |
| | *B. asteroides*, *B. coryneforme*, *B. indicum* [Vasquez et al., 2012] | H |
| Bifido-2 | | H,B |
| | *Bifidobacterium bombi* [Killer et al., 2009] | B |
| Bifido-3 | *Bombiscardovia coagulans* [Killer et al., 2010a] | **B** |
| Bifido-4 | | H,B |
| | *Bifidobacterium bohemicum* [Killer et al., 2011] | B |
| Bifido-X | / | H,B |
| Bacteroidetes | | H,B |
| | *Apibacter adventoris* [Kwong and Moran, 2016b] | H |

H = honeybee, B = bumblebee.
[a] Gamma-2 in Koch and Schmid-Hempel [2011b].
[b] Identity based on a full 16S rRNA gene sequence [Koch and Schmid-Hempel, 2011b].
[c] Identity based on a partial 16S rRNA gene sequence (253 bp) [Meeus et al., 2015].
[d] Phylotype detected by Li et al. [2015].
[e] No sequence available in public databases.
[f] Phylotype detected by Olofsson and Vásquez [2008].

### 2.3.1 *Snodgrassella alvi* and *Gilliamella apicola*

*Snodgrassella alvi* and *Gilliamella apicola* are Gram negative bacteria which correspond to the Beta and Gamma-1 phylotypes, respectively [Kwong and Moran, 2013]. Candidatus *Schmidhempelia bombi* is a bacterium closely related to *G. apicola* (95.8% 16S rRNA gene sequence similarity (1462 bp length)) and its genome was sequenced alongside that of its bumblebee host [Martinson et al., 2014]. Members of this candidate species have not been cultivated yet. *G. apicola* and *S. alvi* are fastidious bacteria and their isolation media (heart infusion and blood agar media) are general and complex and consist of animal organ digests (e.g. heart or brain extract) or blood [Kwong and Moran, 2013]. While *G. apicola* strains are able to ferment different carbohydrates like sucrose and fructose, *S. alvi* oxidises carboxylic acids (e.g. lactate and citrate) [Kwong et al., 2014]. *S. alvi* strains are micro–aerobic while *G. apicola* strains vary from micro–aerobic to facultative anaerobic which potentially reflects their niches in the bee hindgut [Kwong and Moran, 2013].

Although the cultivated representatives of the Beta and Gamma-1 phylotypes currently comprise only one species each (Table 2.2), the nearly full 16S rRNA gene sequences of the Gamma-1 phylotype as present in clone libraries show considerable levels of dissimilarity (up to 5%) indicating that this phylotype consists of multiple species [Koch and Schmid-Hempel, 2011b]. A similar diversity has also been observed for the Firm-4 and Firm-5 phylotypes. In contrast, the sequences of the Beta phylotype are more homogeneous and share more than 98.6% sequence similarity [Koch and Schmid-Hempel, 2011b].

A strong host association has been observed for *S. alvi* with the phylogeny of the bacteria mostly matching the phylogeny of the bumblebee or honeybee host species [Koch et al., 2013]. This association was less strong for *G. apicola.* Furthermore, while *S. alvi* can be detected in all hibernated queens in the lab, some queens lack *Gilliamella. Gilliamella* has also been detected on flowers and in the hive environment (e.g. nectar pots and wax surfaces) [Koch et al., 2013; McFrederick et al., 2016] and therefore seems to be less intimately associated with the honeybee and bumblebee gut [Koch et al., 2013].

### 2.3.2 Lactic acid bacteria and *Bifidobacteriaceae*

In addition to *S. alvi* and *G. apicola*, lactic acid bacteria (LAB) have been isolated from the gut of honeybees and bumblebees [Killer et al., 2013, 2010b,a, 2014; Olofsson and Vásquez, 2008; Olofsson et al., 2014; Vasquez et al., 2012, 2008]. LAB are Gram positive, facultative or strictly anaerobic fermenters. Homo-fermentative LAB ferment hexoses through the Embden-Meyerhof pathway with lactic acid as main end-product while hetero-fermentative LAB produce a variety of end-products (e.g. lactic acid, acetic acid and ethanol) through the 6-phosphogluconate pathway [Khalid, 2011]. Both general and selective media have been used to isolate LAB (e.g. De Man Rogosa Sharpe agar (MRS), Rogosa agar and Tryptic soy broth (TSB) agar) and more specifically *Bifidobacteriaceae* (e.g. Modified tryptone phytone yeast extract agar (MTPY)) from the bee intestinal tract [Killer et al., 2010b; Otti and Schmid-Hempel, 2007; Vasquez et al., 2012]. Multiple *Lactobacillus* species have been isolated from the honeybee crop. Of these, *Lactobacillus kullabergensis*, *Lactobacillus melliventris*, *Lactobacillus kimbladii*, *Lactobacillus helsingborgensis* and *Lactobacillus apis* belong to the Firm-5 phylotype; *Lactobacillus mellis* belongs to the Firm-4 phylotype and *Lactobacillus mellifer* corresponds to the Bin4 phylotype [Olofsson and Vásquez, 2008; Olofsson et al., 2014; Martinson et al., 2011].

Fewer LAB have been isolated from the bumblebee crop compared to the honeybee crop [Olofsson and Vasquez, 2009]. This difference may be explained by the smaller number of bumblebee samples examined or because bumblebees feed their larvae with fresh nectar and do not produce bee bread like honeybees do. Bee bread consists of pollen, saliva, nectar and honey and is fermented by LAB which enhances its preservability. Cultivated representatives of the Firm-5 phylotype have been derived from the bumblebee crop and analysis of about 1000-nucleotide long 16S rRNA gene fragments revealed that these isolates are only distantly related to their honeybee counterparts (there is up to 4.2% dissimilarity) and should be classified as a separate, novel species [Olofsson and Vasquez, 2009]. Isolates corresponding to the Firm-4 phylotype have been described as *Lactobacillus bombi* [Killer et al., 2014]. *Lactobacillus kimchicus* corresponds to the Lacto-5 phylotype and occurs erratically in honeybees and bumblebees [Meeus et al., 2015]. This species is not restricted to the bee gut and has also been isolated from kimchi, a fermented food product [Liang et al., 2011]. Firm-S and Firm-E are two additional LAB phylotypes which have been detected in bumblebees and are identified as *Lactococcus* and *Enterococcus*, respectively.

*Fructobacillus fructosus* and *Lactobacillus kunkeei* represent the Fructobacillus and Lacto-3 phylotypes, respectively. They are fructophilic LAB inhabiting the fructose-rich honey stomach and isolation of these bacteria is promoted by the addition of fructose to the growth medium (e.g. MRS + 2% fructose) [Endo et al., 2012; Endo and Okada, 2008; Endo and Salminen, 2013]. *Fructophilic* LAB prefer fructose over glucose as a growth substrate and occur in fructose-rich niches. They grow on glucose in an aerobic atmosphere and grow poorly on glucose in an anaerobic atmosphere. Growth on glucose in anaerobic atmosphere is enhanced if electron acceptors (e.g. pyruvate or fructose) are added to the growth medium. The need for electron acceptors under anaerobic conditions results from the conversion of acetyl-phosphate to acetate instead of the reduction of acetyl-phosphate to ethanol in the heterofermentative pathway, leading to an insufficient oxidation of NAD(P)H for NAD(P)/NAD(P)H cycling [Endo et al., 2012; Endo and Okada, 2008; Endo and Salminen, 2013]. Recently, a novel species (*Lactobacillus apinorum*) closely related to *L. kunkeei* and thus also corresponding to the Lacto-3 phylotype has been isolated from the honeybee gut [Olofsson et al., 2014]. Although *L. kunkeei* is the most dominant LAB of the honeybee crop [Olofsson and Vásquez, 2008], like *F. fructosus*, it is not restricted to the bee intestine and both species have also been isolated from other fructose-rich environments like flowers and grape juice [Endo and Okada, 2008; Endo et al., 2012].

To stimulate the isolation of fastidious micro–organisms, the natural niche of the isolates can be simulated *in vitro* by adding specific compounds to the isolation media. This approach was adopted by Killer et al. [2010b] who developed a medium supplemented with pollen for the selective isolation of *Bifidobacteriaceae* from the bumblebee gut. Several novel *Bifidobacterium* species and a novel species belonging to a novel genus *Bombiscardovia* have been isolated from the bumblebee gut and have been formally named (Table 2.2). Bifidobacteria isolated from the honeybee crop have been classified into *Bifidobacterium indicum*, *Bifidobacterium asteroides* and *Bifidobacterium coryneforme* [Olofsson and Vásquez, 2008].

### 2.3.3 Other

Cultivated representatives of the Alphaproteobacteria phylotypes (*Bartonella apis* [Kesnerova et al., 2016] and *Parasaccharibacter apium* [Corby-Harris et al., 2014]) and the Bacteroidetes phylotype (*Apibacter adventoris* [Kwong and Moran, 2016b]) are fastidious and were isolated on rich media like heart infusion agar, Colombia blood agar and Sabouraud dextrose agar. These bacteria share the preference of growth in oxygen-reduced atmospheres with most other honeybee and bumblebee gut bacteria. Although *P. apium* is sporadically detected in the bee gut, the species is mainly prolific in royal jelly, hypopharyngeal glands, the crop and larvae [Corby-Harris et al., 2014].

Phylotypes for which no cultivated representatives are available at present are most closely related to some *Pseudomonas*, *Buttiauxella*, *Hafnia*, *Serratia*, *Gluconobacter* or *Lactobacillus* species (Table 2.2). *Pseudomonas* species have been isolated from insect guts by using rich

media like Luria-Bertani (LB) agar. Several studies report on entomopathogenic *Pseudomonas* strains and the genome of *Pseudomonas entomophila*, a species isolated from the gut of *Drosophila*, contains genes coding for insecticidal toxins [Mulet et al., 2012]. *Pseudomonas* strains prefer growth in an aerobic atmosphere which is also the case for *Buttiauxella* strains. *Gluconobacter* species are acetic acid bacteria (AAB) which are obligately aerobic and have been isolated from sugar rich environments like flowers and fruits by using highly nutritious media like glucose yeast extract carbonate agar (GYC agar) [Mamlouk and Gullo, 2013].

### 2.3.4 Enterotypes

An enterotype is a classification of organisms based on their microbial community in the gut. In contrast to honeybees, two gut enterotypes have been observed in bumblebees [Li et al., 2015; Meeus et al., 2015; Parmentier et al., 2016]. Enterotype 1 is dominated by *Enterobacteriaceae* (e. g. Gamma-E1 (corresponding to *Buttiauxella* sp.), Gamma-E2 (corresponding to *Hafnia* sp.) and another Gamma phylotype closely related to *Serratia* sp. while enterotype 2, which is the most prevalent, is dominated by the Gamma-1 and Beta phylotypes. *Lactobacillus* species are abundant in both enterotypes.

Bumblebee queens can loose typical gut bacteria during hibernation [Koch et al., 2013]. The guts of these queens may be more prone to colonisation by bacteria from the environment like members of the *Enterobacteriaceae* which might explain the occurrence of two enterotypes in bumblebees [Li et al., 2015; Meeus et al., 2015].

### 2.3.5 Localisation

Most gut bacteria are located in the ileum and rectum [Kwong and Moran, 2016a; Martinson et al., 2012]. The crop with its low pH, enzymatic activity and frequent content renewal and the midgut with its digestive enzymes and continuous shedding of the peritrophic membrane are probably more hostile environments for microbial colonisation [Martinson et al., 2012]. The crop mainly contains members of the genera *Lactobacillus* and *Bifidobacterium* which form biofilms on the crop surface [Vasquez et al., 2012]. The midgut contains low numbers of *Snodgrassella* [Martinson et al., 2012] while the ileum with its large invaginations is dominated by *Snodgrassella* and *Gilliamella*. *Snodgrassella* is directly associated with the ileum intima while *Gilliamella* fills the rest of the invaginations [Engel et al., 2012; Martinson et al., 2012]. Firm-5 is detected in the ileum lumen and dominates in the rectum [Martinson et al., 2012]. *Frischella perrara* corresponds to the Gamma-2 phylotype and is a species which only colonises a restricted region in the honeybee gut called the pylorus [Engel et al., 2015, 2013]. This species grows optimally under anaerobic conditions and causes scab formation before colonisation probably by inducing the honeybee immune system.

### 2.3.6 Colonisation

Larvae and newly emerged workers contain few bacteria and adults obtain their typical gut composition approximately four days after emergence [Billiet et al., 2016; Kwong and Moran, 2016a; Martinson et al., 2012]. Adult bees can acquire gut micro–organisms in several ways. The bees can become colonised with the typical bee gut microbiota while emerging from the cocoon. They also obtain gut bacteria through contact with older individuals and the nest or through contact with the environment by foraging [Billiet et al., 2016; Martinson et al., 2012]. Social contact greatly facilitates the colonisation of Beta, Gamma-1, Firm-5 and Firm-4 bacteria but is not strictly necessary [Billiet et al., 2016; Koch et al., 2013]. However, contact with nest mates was required for the colonisation of the gut with *Bifidobacteriaceae.* In addition, the numbers and members of LAB in the crop of honeybees increases with foraging activity [Vasquez et al., 2008]. Therefore, some LAB are probably obtained from the environment.

The gut microbiota of bumblebees also changes while the colony ages and is characterized by a decrease in the relative abundance of the Beta, Gamma-1 and Bifido-3 phylotypes and an increase in the relative abundance of the Firmicutes and Bacteroidetes phylotypes towards the end of the season [Koch et al., 2012].

In commercial bumblebee nests, the gut microbiota composition is a subset of that observed in wild bumblebees and is dominated by the core bacteria (Beta, Gamma-1, Firm-5, Bifido-3) with a higher relative abundance of the Beta phylotype [Meeus et al., 2015].

### 2.3.7 The gut microbiota composition of stingless bees and orchid bees

The gut microbiota composition of honeybees and bumblebees differs from that of stingless bees which is the third social corbiculate bee tribe. In stingless bees the most abundant phylotypes are a *Ralstonia*, two *Pantoea* and a Rhodospirillales phylotype [Leonhardt and Kaltenpoth, 2014]. In addition, also the Firm-4 and Firm-5 phylotypes are detected and several other phylotypes corresponding to species of *Lactobacillus*, *Leuconostoc*, *Aerococcus*, *Streptococcus* and *Bifidobacterium*, of which some are shared with the honeybees and bumblebees [Koch et al., 2013; Vasquez et al., 2012]. *Gilliamella* nor *Snodgrassella* species have been detected in stingless bees. Similarly, they were not detected in orchid bees which is the fourth social corbiculate bee tribe [Koch et al., 2013]. Although, no thorough studies of the gut microbiota of orchid bees have been reported, a phylotype related to the Alpha 2.2 phylotype and characterized by up to 4% 16S rRNA gene sequence dissimilarity, was highly abundant in three analysed orchid bee species. Furthermore, a second unknown Alphaproteobacterium phylotype and a Gammaproteobacterium phylotype closely related to *Yokenella regensburgi* were detected [Koch et al., 2013].

## 2.4 Bumblebee-gut microbiota interactions

As mentioned above, the gut microbiota composition of honeybees and bumblebees is similar and is dominated by up to five taxa which are consistently present. A similar gut microbiota between related hosts can be explained by co-speciation or by similar gut environmental conditions and gut micro–organism acquisition modes [Moran and Sloan, 2015].

In honeybees and bumblebees, there is evidence of co-speciation of *Snodgrassella* and *Gilliamella* [Koch et al., 2013; Kwong and Moran, 2015]. The intimate association of honeybees and bumblebees with these two symbionts also became apparent from the specificity of the association and the specialization of the bacteria to their hosts [Koch et al., 2013; Kwong and Moran, 2015; Kwong et al., 2014]. Specificity of gut microbiota-host associations is defined as the restriction of a micro–organism to a particular set of host species [Kwong and Moran, 2015]. This differs from specialization which is defined as the adaptation of a micro–organism to a particular set of hosts and adaptation of the host to the micro–organism [Kwong and Moran, 2015]. Host specialisation was shown for *Snodgrassella* by competition and transplantation assays as higher levels of colonisation were observed when bees were fed with their native *S. alvi* strains and native strains were able to become dominant despite a numerical disadvantage to non-native strains [Kwong and Moran, 2015].

Apart from co-speciation, a similar gut microbiota can result from similar gut environmental conditions between hosts. Hosts determine which micro–organisms are able to settle in the gut by two mechanisms, called tolerance and resistance [Engel and Moran, 2013]. Tolerance is defined as the ability of the host to reduce the negative impacts of a particular microbial load and resistance is the ability to reduce the microbial load. An example of a tolerance mechanism in bees is the shedding of the peritrophic membrane. This shedding does not reduce the bacterial load but impedes contact of the micro–organisms with the epithelial cells. Resistance can be obtained by the low pH in the honey stomach and the immune response [Engel and Moran, 2013]. The bee gut microbiota can undoubtedly affect bee health and relationships can be either commensal (beneficial for the micro–organism without harming the host), mutualistic (beneficial for both the micro–organism and the host) and parasitic (harmful for the host). The precise interactions of the honeybee and bumblebee hosts with most of their gut microbionts and the interactions between these gut microbionts remain elusive. However, different functions have been assumed ranging from pathogen defense, digestion of nutrients, degradation of toxic compounds and nutrient provisioning [Cariveau et al., 2014; Engel et al., 2012; Forsgren et al., 2009; Koch and Schmid-Hempel, 2011b; Koch et al., 2012; Zheng et al., 2016].

### 2.4.1 Defense against pathogens

Gut symbionts can mediate defense against pathogens through exploitation competition (direct competition for nutrients), exclusion competition (efficient colonisation of the epithelium), immune-mediated competition (inducing an immune response which differentially affects microbial strains) and interference competition (direct inhibition through secretion of toxins or changing the pH) [Koch et al., 2013]. LAB are well-known for the production of hydrogen peroxide, various other antimicrobial compounds and short-chain fatty acids that lower the pH of the environment. LAB isolated from the honeybee crop were able to inhibit growth of the honeybee pathogens *P. larvae* and *M. plutonius in vitro* and *in vivo* [Forsgren et al., 2009; Vasquez et al., 2012]. Crop LAB (Figure 2.1) and ileal *Snodgrassella alvi* strains are attached to the intestinal wall and are embedded in extracellular polymeric substances (EPS) [Engel et al., 2012; Martinson et al., 2012; Vasquez et al., 2012]. These aggregates of micro–organisms and EPS (e.g. polysaccharides, nucleic acids, proteins and lipids) are called biofilms and hamper colonisation of the intestinal wall by other potentially harmful micro–organisms.

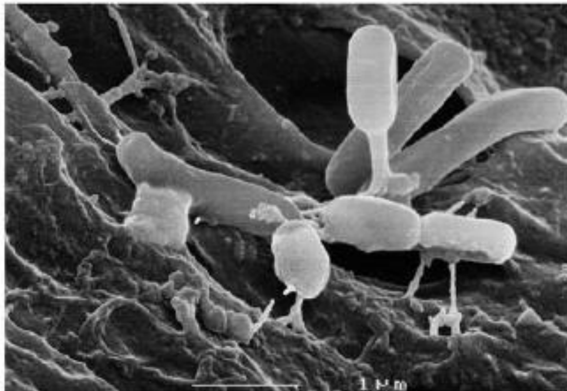

**Figure 2.1:** *In vitro* visualization of lactobacilli attached to the wall of a honeybee crop using SEM. Figure from Vasquez et al. [2012].

The gut microbial composition is significantly different in bumblebees infected with *C. bombi*: *C. bombi* is negatively associated with abundance of *Gilliamella* and *Snodgrassella* and is positively associated with abundance of the Alpha-2.2 phylotype and other non-core bacteria [Cariveau et al., 2014]. *Snodgrassella* biofilms in the ileum might also protect against *C. bombi* infection [Engel et al., 2012]. The richness of noncore taxa and low numbers of gut bacteria associated with *C. bombi* infection may represent dysbiosis (i.e. an unbalance of the gut microbiota) of the bumblebee gut and may be indicative of an unhealthy bumblebee [Cariveau et al., 2014; Hamdi et al., 2011]. The host microbiota can be seen as an extended immune system important in defense against pathogens. Bumblebees are more protected against *C. bombi* infections if they are fed with faeces of nest mates (Figure 2.2) and the susceptibility to *C. bombi* infection not only depends on the parasite and host genotype but also on the 'microbiota type' [Koch and Schmid-Hempel, 2011a; Koch et al., 2012].

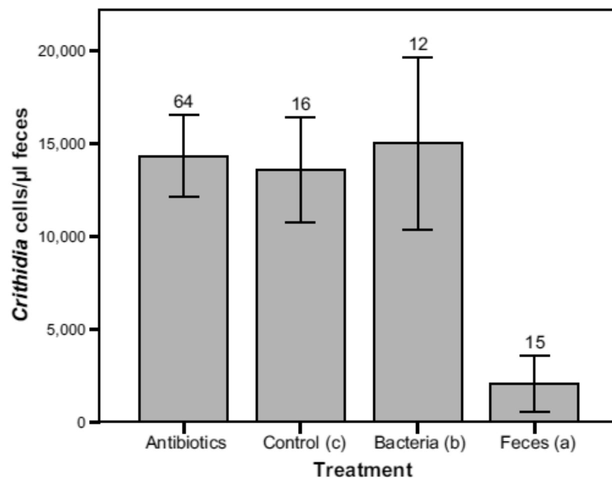


**Figure 2.2:** Comparison of the number of *C. bombi* cells per µl of faeces at 7 days post infection for three treatment groups (bumblebees fed with faeces (a); a *Gilliamella* strain (b) or sugar water (c)) and antibiotics-fed bumblebees. The sample size of each group is listed above the bars. Figure from Koch and Schmid-Hempel [2011a].

### 2.4.2 Detoxification and nutrient provisioning

The bee gut microbiota helps with the digestion of nutrients from nectar and pollen [Engel et al., 2012; Lee et al., 2015]. The sugars present in nectar can be fermented by LAB, AAB and *Gilliamella* strains with the formation of short-chain fatty acids. The latter can be taken up by epithelial cells and account for a large proportion of the bee's energy sources [Engel et al., 2012; Lee et al., 2015]. Some *Gilliamella* strains are able to digest pectin which is a compound of the pollen wall and which is toxic to bees. These strains may therefore contribute to the weakening of the pollen wall and to detoxification [Engel et al., 2012; Zheng et al., 2016]. In addition, some *Gilliamella* strains digest toxic sugars (e.g. mannose, arabinose, xylose and rhamnose) sometimes present in nectar and pollen [Engel et al., 2012; Zheng et al., 2016]. Gut micro–organisms may also play a role in the degradation of pesticides and herbicides in nectar and pollen and might also provide essential nutrients to the insect. Insects obtain essential vitamins and amino acids mainly from pollen, however, the amino acid and vitamin composition varies with the type of pollen collected. Essential amino acids for insects include aromatic, aliphatic, sulfur containing and basic amino acids, however, tryptophan and phenylalanine are often lacking in pollen [Boudko, 2012; Cook et al., 2003; Roulston and Cane, 2000]. Essential vitamins for insects are water-soluble vitamins B and C and fat-soluble vitamins A and E. Pollen is rich in water-soluble vitamins and especially B vitamins [Chapman, 1969]. The production of amino acids and vitamins by gut micro–organisms might supplement the diet of bees which can become essential when little high quality pollen is available.

## 2.5 Microbial resource management to improve the health of bees

The gut microbiota is often referred to as a complex ‘superorganism’ with an essential role in health and development of the host [Crotti et al., 2012]. Gut micro–organisms are responsible for a large number of functions ranging from efficient digestion of food, provisioning of essential nutrients and detoxification of ingested compounds to pathogen defense. Maintenance of these beneficial functionalities in the gut is essential and loss of functional potential due to changes in microbiota composition is referred to as dysbiosis [Crotti et al., 2012]. Microbial resource management (MRM) in this context attempts to influence the gut microbiota to restore the native microbiota composition but especially the native microbiota functionality [Crotti et al., 2012; Verstraete et al., 2007]. The practical implementation of MRM is often very complex and a thorough knowledge of the microbial community is required [Crotti et al., 2012; Verstraete et al., 2007]. A microbial gut community can be influenced by probiotics, prebiotics or a combination of both (synbiotics). A probiotic is defined as “a mono- or mixed culture of live micro-organisms which, when applied to man or animals, beneficially affects the host by improving the properties of indigenous microflora” [Havenaar et al., 1992] while a prebiotic is defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” [Gibson and Roberfroid, 1995].

LAB are the most often used probiotic bacteria mainly because they produce antioxidants and antimicrobial compounds, induce the immune system and are generally regarded as safe. They are tolerant to a low pH which allows efficient passage of living cells through the stomach into the gut [Ljungh and Wadstrom, 2006]. Also in honeybees the effect of LAB as probiotics has been investigated and adding a mixture of LAB from the honey stomach to honeybee larvae significantly decreased the number of larvae infected with *P. larvae* [Forsgren et al., 2009; Killer et al., 2013; Vasquez et al., 2012]. Projects like MICRO4BEE (patent PCT/IB2012/053459) and BeeProbio (http://www.crsad.qc.ca/) investigate the effect of commercial probiotics mainly consisting of *Lactobacillus* and *Bifidobacterium* species on bee health. Feeding of honeybee larvae with the MICRO4BEE probiotic product was associated with a higher survival upon infection with *P. larvae* due to direct antagonism with the pathogen, induction of the immune system and restoration of the optimal microbiota (patent PCT/IB2012/053459). Although positive effects on honeybee health have been shown for some of these commercial probiotics, the administration of these products and specific strains must be well-considered. Ptaszyńska *et al.* reported a higher susceptibility of honeybees to *N. ceranae* infections and a shorter lifespan of honeybees when fed with commercial probiotics based on *Lactobacillus rhamnosus* strains [Ptaszynska et al., 2016]. However, significantly lower levels of *N. ceranae* were detected when honeybees were fed with bifidobacteria and lactobacilli isolated from honeybees [Baffoni et al., 2016].

# Part II

# Aims and Outline

# Aims and Outline

As discussed in **Chapter 1** bumblebees are efficient generalist pollinators which are adapted to temperate climates. Commercial bumblebee colonies are used in agriculture worldwide for the successful pollination of greenhouse crops. The prevalence of pathogens in wild bumblebees is higher in the proximity of greenhouses with commercial colonies and these colonies may act as a pathogen reservoir.
Different studies outlined in **Chapter 2** showed that the bumblebee gut microbiota contributes to its hosts' health and indicated that the bumblebee gut microbiota consists of several novel species of bee specific taxa with unknown functionality.
The accurate identification and characterisation of the bumblebee gut microbiota is essential fundamental research which will help to understand the physiology of bumblebees. It is also application–oriented research with a potential to exploit beneficial strains in commercial rearing facilities. In the present study we aim to isolate and identify the bumblebee gut microbiota. Subsequently, we aim to characterize the isolates obtained and to explore their biotechnological potential.

The diversity of the bumblebee gut microbiota has mainly been studied by cultivation–independent approaches. Because these studies lack taxonomic resolution, we hypothesize that this diversity is considerably underestimated. In addition, the availability of microbial isolates facilitates the analysis of their functional potential. Hence, **a first goal** is therefore to properly and thoroughly inventorise the cultivable bumblebee gut microbiota through an extensive isolation campaign, coupled to state-of-the-art species–level identification of isolates through a combination of MALDI-TOF mass spectrometric dereplication followed by sequence analysis of 16S rRNA and/or protein encoding genes of selected isolates. Bumblebees of *B. lucorum*, *B. terrestris*, *B. pascuorum* and *B. lapidarius* were sampled at five locations (two botanical gardens, two urban and one rural location) to take into account potential variations in the gut microbiota composition associated with bumblebee species and sampling location.

As described in Chapter 2, the bumblebee gut microbiota consists of several novel species. **A second goal** is to formally describe and name these novel species by performing the required polyphasic taxonomic studies and by making reference cultures of these novel species available to the scientific world through their deposit in public culture collections.

Based on the results of reported metagenomics and *in vivo* studies, we hypothesize that the bumblebee gut microbiota has the potential to contribute to bee health. **A third goal** is therefore to explore the functional potential of the isolates through pathogen inhibition assays and a pectin degradation assay. To this end, an *in vitro* assay was developed to examine the isolates' capability to inhibit growth of the bumblebee parasite *C. bombi*. In addition, a well diffusion assay was implemented to analyse their potential to inhibit growth of the honeybee pathogens *P. larvae* and *M. plutonius* and the bee pathogen *A. apis*. These pathogens occur in bumblebees but do not cause infection which might be explained by a different bee physiology or by the gut microbiota. The induction of an immune response is associated with a high energy cost for the bees. Gut micro-organisms capable of degrading pectin, which is a compound of the pollen wall and toxic to bees, may therefore stimulate the immune system by mediating the release of undigested nutrients and may reduce pectin related toxicity.

The data generated in the frame of the present study are presented in chapters 3 and 4. In **Chapter 3** we report an extensive isolation and identification campaign and the results of the functional assays. **Chapter 4** presents the formal descriptions of novel bacteria isolated from bumblebee gut samples. Section 4.1 comprises the description of *Bombella intestini* gen. nov. sp. nov. which represents the Alpha-2.2 phylotype. Sections 4.2 and 4.3 present the descriptions of novel LAB including *Lactobacillus bombicola* sp. nov., a species belonging to one of the core bumblebee gut microbiota taxa (Firm-5), *Weissella bombi* sp. nov., *Bifidobacterium commune* sp. nov. and *Convivina intestini* gen. nov. sp. nov. Section 4.4 encompasses the formal description of a rare, although specific Bacteroidetes member of the bumblebee gut microbiota, *Apibacter mensalis* sp. nov. and a description of its functional potential based on the genome sequence analysis of its type strain. Finally, four novel *Gilliamella* species are described in section 4.5 and their potential to degrade pectin and toxic sugars was explored by analysis of their genome sequences.

To conclude, **Chapter 5** presents a general discussion of the results and provides some future perspectives.

# Part III

# Experimental work

# CHAPTER 3

## Diversity and functionality of the cultivable bumblebee gut microbiota

The gut microbiota of bumblebees has mainly been studied by cultivation–independent approaches like 16S rRNA amplicon sequencing. However, the availability of microbial isolates greatly facilitates the exploration of its functional potential. Chapter 3 describes an extensive isolation campaign of the gut microbiota of four *Bombus* species sampled at five locations. A large set of microbial isolates was obtained and identified to the species level through a combination of MALDI–TOF MS dereplication and sequence analysis of 16S rRNA and/or protein encoding genes of representative isolates from each MALDI-TOF MS cluster.

Subsequently, the functional potential of the isolates was analysed *in vitro*. A well diffusion assay was implemented to investigate the isolates' ability to inhibit growth of the honeybee pathogens *P. larvae*, *M. plutonius* and *A. apis*. Furthermore, a 96–well assay was developed to test the ability of the isolates to inhibit the bumblebee parasite *C. bombi*. Finally, the ability of the isolates to mediate digestion of pollen was analysed through *in vitro* pectin degradation assays. Results of these functional assays are described in the present chapter.

## 3.1 Large-scale cultivation of the bumblebee gut microbiota reveals an underestimated bacterial species diversity and functionality

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**Author contributions:** JP, PV, IM and GS conceived the study. JP and PV wrote the manuscript. JP performed the experiments and data-analysis. AP performed the data-analysis of the 16S rRNA amplicon sequencing. RS provided the *Crithidia bombi* strains. IM, AP, RS and GS proofread the manuscript.

### 3.1.1 Abstract

A total of 1940 isolates from gut samples of 60 bumblebees representing *Bombus pascuorum*, *Bombus terrestris*, *Bombus lucorum* and *Bombus lapidarius* was collected and identified through state-of the-art taxonomic methods. The bacterial species diversity in these *Bombus* species exceeded that suggested by phylotype analysis through 16S rRNA amplicon sequencing considerably and revealed that *B. pascuorum* and *B. terrestris* had a unique microbiota composition, each. Representatives of most phylotypes reported earlier and detected in the present study were effectively isolated, and included several novel bacterial taxa and species reported for the first time in the bumblebee gut. The functional potential of 233 isolates was explored by pectin degradation assays and growth inhibition assays against the honeybee pathogens *Paenibacillus larvae*, *Melissococcus plutonius* and *Ascosphaera apis* and the bumblebee parasite *Crithidia bombi*. While inhibitory activity against each of these pathogens was observed, only one single culture was able to degrade pectin and polygalacturonic acid *in vitro*.

### 3.1.2 Introduction

The gut microbiota of honeybees and bumblebees is similar and has mainly been studied by cultivation independent methods [Martinson et al., 2011; Meeus et al., 2015; Mohr and Tebbe, 2006; Moran et al., 2012]. Phylotypes that include the genera *Snodgrassella* (Beta), *Gilliamella* (Gamma-1) and *Lactobacillus* (Firm-4/Lacto-2 and Firm-5/Lacto-1) are commonly present [Martinson et al., 2011; Meeus et al., 2015; Mohr and Tebbe, 2006; Moran et al., 2012] but also members of the Alphaproteobacteria, Actinobacteria and additional Gammaproteobacteria have been detected [Li et al., 2014a; Cariveau et al., 2014; Moran et al., 2012]. Cariveau et al. [2014] and Koch et al. [2011, 2013] reported that gut microbiota composition and richness differed significantly between certain bumblebee species and that the gut microbiota composition was consistent over different locations [Cariveau et al., 2014; Koch and Schmid-Hempel, 2011b; Koch et al., 2013]. This geographic consistency combined with endosymbiont specificity revealed a strong functional dependence. Genome sequence analyses confirmed host specific specialization of both *Gilliamella apicola* and *Snodgrassella alvi* and suggested a long-term host association with variations in genes coding for host-interaction factors as the underlying mechanism for host specificity [Kwong et al., 2014].
Several studies demonstrated that bee specific gut microbiota contribute to maintaining bee health through pathogen inhibition and pectin degradation [Koch and Schmid-Hempel, 2011a; Zheng et al., 2016; Vasquez et al., 2012; Engel et al., 2012]. The functional potential of these micro-organisms however may be strain specific as a considerable part of bacterial ecological adaption is encoded in the accessory, rather than core genome [Lapierre and Gogarten, 2009]. In addition, the species diversity of the bumblebee gut microbiota as revealed by 16S rRNA amplicon sequencing may be considerably underestimated as typically fragments of only 250 base pairs are sequenced which limits the taxonomic resolution to that of the genus or even family level [Clarridge, 2004]. The availability of microbial isolates will facilitate proper evaluation of the functional potential of the gut microbiota. To generate a more complete picture of the bacterial species and functional diversity, we aimed to collect a comprehensive set of gut isolates from 60 bumblebees belonging to four *Bombus* species, to accurately identify all isolates to the species level, and to analyse their functional potential.

### 3.1.3 Materials and Methods

Sampling and identification of bumblebees and preparation of cell suspensions

*Bombus pascuorum* (n = 16), *Bombus terrestris* (n = 19), *Bombus lucorum* (n = 6) and *Bombus lapidarius* (n = 19) bumblebees were caught between August 2013 and September 2013 or between July 2014 and September 2014 in five locations near Ghent, Belgium (1 = Bourgoyen Ghent [51.06840°NL/3.685100°EL], 2 = Coupure Ghent [51.05130°NL/3.706°EL], 3 = Gentbrugge [51.0462°NL/3.7608°EL], 4 = Ledeganck botanical garden Ghent [51.0368°NL/ 3.7221°EL] and 5 = Den blakken botanical garden Wetteren [51.0086°NL/3.899100°EL]), and were identified by their colour pattern. Identification results as *B. lapidarius*, *B. terrestris*

and *B. lucorum* were confirmed through cytochrome oxidase I (COI) gene sequence analysis [Carolan et al., 2012]. The COI gene was amplified as described by Carolan et al. [2012] after Chelex® 100 resin (Bio-Rad) DNA-extraction from two bumblebee legs which were grinded with a micropestle [Walsh et al., 1991]. The bumblebees were immobilized at -20°C for 10 minutes and surface sterilized with 2.5% Umonium38® Master (Laboratoire Huckert's International, Brussels, Belgium) before dissection. The gut (crop, midgut and hindgut) was dissected by cutting along the ventral surface of the abdomen and was homogenized in 250 µl saline (0.1% peptone, 0.1% Tween 80, 0.85% sodium chloride) with a sterile micro-pestle [Olofsson and Vásquez, 2008]. These cell suspensions were divided into 2 tubes comprising 125 µl each, after which 125 µl of a 10% dimethylsulfoxide (DMSO) solution was added to each tube. Cell suspensions were homogenized and stored at -80°C using a Nalgene® Mr. Frosty™ Freezing container until further use.

Isolation of bumblebee gut bacteria and dereplication

Cell suspensions were thawed at 37°C and centrifuged at 6800 X g for 15 minutes at 4°C. The supernatant was discarded to remove DMSO and pellets were suspended in 1 ml of physiological water (0.85% sodium chloride) and serially diluted to $10^{-4}$. The resulting cell suspensions were plated on de Man, Rogosa and Sharpe (MRS) agar (Oxoid) supplemented with 0.1% sorbic acid pH 5.7, modified trypticase phytone yeast extract (MTPY) agar without mupirocin [Rada and Petr, 2000], M144 agar (consisting of 23 g/l special peptone, 1 g/l soluble starch, 5 g/l sodium chloride, 0.3 g/l cysteine hydrochloride, 5 g/l glucose and 15 g/l agar) and all culture (AC) agar supplemented with 10 ppm cycloheximide (Sigma-Aldrich) [Ha et al., 1995] and incubated aerobically for MRS agar, anaerobically (80% $N_2$, 10% $H_2$, 10% $CO_2$) for MRS, MTPY and M144 agar and micro-aerobically (80% $N_2$, 15% $CO_2$ and 5% $O_2$) for AC agar at 37°C. After 5 days, colonies were picked and transferred to the respective isolation media. Third generation axenic isolates were dereplicated as described earlier [Ghyselinck et al., 2011] by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) followed by curve-based data analysis using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium).

Identification of isolates

Representative isolates of each MALDI-TOF MS profile cluster were selected for tentative identification by 16S rRNA gene sequence analysis [Ghyselinck et al., 2011]. Most isolates were identified as lactic acid bacteria (LAB) and bifidobacteria and were more accurately identified to the species level through *pheS* and *hsp60* sequence analysis, respectively, based on a reported 3% intra-species divergence [Zhu, 2003; Naser et al., 2007]. In the frame of earlier taxonomic studies, *Bombella*, *Apibacter* and *Gilliamella* isolates were further analysed and classified through *groEL* sequence analysis, multi-locus sequence analysis and average nucleotide identity values, respectively [Praet et al., 2016; Li et al., 2014a; Praet et al., 2017]. Yeast isolates were identified at the species level through 26S rRNA gene sequence analysis, based on the reported 1% intra-species divergence for ascomycetous yeasts

[Kurtzman and Robnett, 1998; Lopandic et al., 2006]. When lower rRNA sequence identity values were obtained, isolates were considered identified at the genus level only. Yeast DNA was extracted through a single-tube lithium acetate sodium dodecyl sulphate lysis protocol [Lõoke et al., 2011]. The 26S rRNA sequences were determined as previously described [Cocolin et al., 2000] and PCR was carried out in 25 µl reaction volumes using 2.5 µl 10x PCR-buffer (Applied Biosystems), 2.5 µl dNTPs (2 mM each, Applied Biosystems), 0.25 µl LROR and LR6 primers (10µM), 0.5 µl Taq polymerase (1U/µl, Applied Biosystems) and 2.5 µl DNA-extract [Vilgalys and Hester, 1990; Rehner and Samuels, 1994].
All remaining isolates were considered identified at the species level if the complete 16S rRNA gene sequence showed at least 98.65% identity to the sequence of the respective type strain [Kim et al., 2014]. When lower rRNA sequence identity values were obtained, isolates were considered identified at the genus level only. EzTaxon-e [Kim et al., 2012] was used for analysis of the 16S rRNA gene sequences, NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST) for the analysis of the *pheS* and *hsp60* gene sequences and FungalBarcoding (http://www.fungalbarcoding.org/) for the analysis of the 26S rRNA gene sequences. Per bumblebee one isolate of each species was selected for subsequent functional analyses.

Pathogen inhibition assays

A well diffusion assay based on the protocols used by Killer et al. [2013] and Nanda and Saravanan [2009] was performed to test the ability of the isolates to inhibit growth of *Paenibacillus larvae*, *Melissococcus plutonius* and *Ascosphaera apis*. *P. larvae* strains LMG 9820$^T$, R-54592 and R-54334 and *M. plutonius* LMG 20360$^T$ were cultivated aerobically and anaerobically, respectively, for two days on AC agar at 37°C. *A. apis* MUCL 34668 was cultivated aerobically for two days on AC agar at 28°C. The pathogens (approximately 1x$10^7$ cells/ml for *P. larvae* and *M. plutonius*, and OD600 = 0.5 after filtering with a Falcon® 40 µm cell strainer (Corning) for *A. apis* as described by Botterel et al. [2008]) were inoculated onto AC agar plates (55 mm diameter) as described by Nanda and Saravanan [2009]. Subsequently, holes with a diameter of 5 mm were punched aseptically into the middle of the agar plates using the reverse end of a pipetting tip (20 µl LTS tips GPS-L10 Mettler Toledo) and were inoculated with 50 µl of isolate cultures. The latter cultures were obtained after growth in AC broth (pH 6 or 7) for two days at 37°C in the respective aerobic, micro-aerobic or anaerobic conditions. AC broth was used as a negative control and *Lactobacillus apis* LMG 26964$^T$ as a positive control. After two to four days of incubation the inhibition zones were measured with a Mauser digital 2 micrometer. All inhibition tests were performed in duplicate and reproducibility was checked during two independent experiments. The well diffusion assays were also repeated with cell-free supernatant obtained by filtering cell cultures through 0.2 µm filter units (Whatman™) or by centrifugation at 15000 rpm for 15 minutes.

To determine the capacity of the test isolates to inhibit growth of *C. bombi*, a 96-well microtiter plate assay was developed. Five *C. bombi* strains, i.e. 08.037, 08.132, 08.169, 08.246, 08.281 [Salathé et al., 2012] were grown in FP-FB broth as described by Salathé et al. [2012].

*Crithidia* cells were counted in a counting chamber Bürker BLAUBRAND® after dilution in 2% formaldehyde to prepare suspensions of $10^7$ cells/ml. One yellow loop (Looplast®) of bumblebee gut isolate cells was inoculated into 3 ml FP-FB broth and after incubation for 5 days the supernatant was filter-sterilised with 0.2 µm filter units (Whatman™). Each well of a Greiner Bio-One 96-well plate (item No: 655161) was filled with 20 µl of a *C. bombi* cell suspension ($10^7$ cells/ml), 80 µl FP-FB medium and 100 µl supernatant. As a negative control 180 µl FP-FB medium was added to 20 µl of *C. bombi*. Tests were performed in triplicate and plates were incubated for 5 days at 28°C in jars (Oxoid™ AnaeroJar™) with a $CO_2$ Gen™ 2.5L Atmosphere Generating Systems bag (Thermo Scientific). Growth inhibition was inspected visually and was confirmed by OD600 measurement with path-length correction by using a SpectraMax PLUS384 spectrophotometer (Molecular Devices). Inhibition mechanisms were analysed by repeating the assay with cell-free supernatant at pH 5.8 and 7 to verify the effect of pH and organic acids, respectively [Kormin et al., 2001]. In addition, cell-free supernatant at pH 7 was treated for two hours at 37°C with catalase (1 mg/ml Merck) to eliminate the effect of hydrogen peroxide and with proteinase K (1 mg/ml Serva) and porcine pancreatic trypsin (1 mg/ml Sigma) to eliminate the effect of bacteriocins. After two hours enzyme activity was inactivated by heating the supernatant for three minutes at 100°C [Kormin et al., 2001].

Pectin degradation assay

To test the ability of the isolates to degrade pectin or polygalacturonic acid (PGA), isolates were streaked onto MP-7 agar plates (HiMedia Laboratories) at pH 5 or 7 containing either 5 g pectin from citrus peel (Sigma) or polygalacturonic acid (Sigma). After two days of incubation, 1% hexadecyltrimethylammonium bromide (CTAB, Fluka) was poured onto the plates. After 20 minutes, pectin or PGA degradation zones were measured with a Mauser digital 2 micrometer. The ability to degrade PGA was also tested by growing isolates onto AC agar for two days and pouring the plates with an overlay agar containing polygalacturonic acid as described by Engel et al. [2012]. *Dickeya chrysanthemi* LMG 2804$^T$ was used as a positive control, as a negative control this strain was streaked onto MP-7 agar plates without pectin.

Draft genome sequence analysis

The genome of *Weissella bombi* LMG 28290$^T$ was sequenced using the Illumina HiSeq 2000 platform. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. Raw Illumina sequence data was passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artifacts. The following steps were then performed for assembly: filtered Illumina reads were assembled using Velvet (version 1.2.07 [Zerbino and Birney, 2008]), 1–3 kb simulated paired end reads were created from Velvet contigs using wgsim (version 0.3.0 (https://github.com/lh3/wgsim)) and Illumina reads were assembled with simulated read pairs using Allpaths–LG (version r4665 [Gnerre and MacCallum, 2011]). The genome

accession number for *W. bombi* LMG 28290$^T$ is ERS1461568. The genomes of *Streptomyces albidoflavus* R-53649 and *Bacillus licheniformis* R-53713 were sequenced using the Illumina HiSeq 4000 platform. Sequence assembly of raw reads into contigs was performed with CLCgenomics workbench 7. The quality of the assemblies (draft genomes) was assessed with Quality Assessment Tool for Genome Assemblies (QUAST (http://quast.bioinf.spbau.ru/)). Genome annotation was performed by RAST [Aziz et al., 2008] and the genomes were screened for the presence of secondary metabolite biosynthesis genes by antiSMASH [Weber et al., 2015]. The genome accession numbers for *S. albidoflavus* R-53649 and *B. licheniformis* R-53713 are ERZ393431 and ERZ392950, respectively.

Statistical analyses

RStudio (version 0.99.491) was used to visualise the relationship between the microbial presence/absence profiles of the bumblebee samples [Team, 2015]. A Bray-Curtis dissimilarity matrix was calculated based on these profiles and used to perform a non-metric multidimensional scaling (NMDS). Two samples were excluded from the analysis as these samples were considered outliers based on a NMDS analysis (data not shown). A one-way analysis of similarity (ANOSIM) was performed with the Past software (version 3.14) on the Bray-Curtis dissimilarity matrix to test if the microbial community differs significantly between bumblebee species or sampling locations and Bonferroni-corrected p-values were calculated for the pairwise tests. SIMPER (Similarity Percentage) was performed with the Past software to assess which species are primarily responsible for observed differences between groups. The iNEXT package in RStudio was used to plot rarefaction curves by using the species richness estimator.

Illumina MiSeq sequencing

Intestinal suspensions were pooled according to bumblebee species and centrifuged at 6800 X g for 15 minutes. The supernatant was discarded and DNA was extracted from a 40 mg pellet as described by Meeus et al. [2015], using the STRATEC Invisorb Spin Tissue Mini Kit. Samples were prepared for sequencing of the variable V3 and V4 region of the 16S rRNA gene. This region was amplified with the universal 341F and 806R primers and the 2x KAPA HiFi HotStart ReadyMix with the following thermal cycling profile: an initial denaturation at 95°C for 3', 25 cycles of denaturation at 95°C for 30", annealing at 55°C for 30", extension at 72°C for 30" and a final extension at 72°C for 5'. Dual indices and Illumina sequencing adaptors were attached with a second PCR amplification step using the Nextera® XT Index Kit (Illumina) following the same cycling conditions for 8 cycles. After each PCR amplification step, a PCR cleanup was executed using the Agencourt AMPure XP beads (Beckman Coulter) following the manufacturer's protocol. The amplicon concentration was quantified using the Quantus™ Fluorometer (Promega) and the amplicons were pooled in equimolar concentrations. The library was sequenced on MiSeq using paired 300-bp reads and MiSeq v3 reagents following manufacturer's protocol (BaseClear).
The MiSeq run output contained 142,894 demultiplexed paired-end reads for the four pooled

bumblebee samples in total. These raw data are available under the study accession number PRJEB18273. The paired-end reads were merged with the PEAR software allowing a minimal overlap size of 20 base pairs, a minimal read length of 250 base pairs and zero uncalled bases in a read. The obtained reads were subsequently trimmed by PEAR. If the quality scores of two consecutive bases was strictly less than the specified quality threshold of 30, the rest of the read was trimmed. The minimum read length allowed after trimming was 200 base pairs. The remaining 142,633 reads were further analysed with the mothur software v.1.35.1 (last updated 03 October 2015) [Schloss et al., 2009], following the standard operating procedure available on the website (http:///www.mothur.org/wiki/MiSeq–SOP). Sequences without ambiguous bases and with a length of approximately 460 base pairs were selected. This resulted in 142,616 reads of which 78,927 were unique sequences. Sequences were further denoised by clustering all sequences with 2 mutations per 100 base pairs. The remaining 31,081 unique sequences were screened for possible chimeras using the UCHIME algorithm, which excluded 4,599 potential chimeras [Edgar et al., 2011]. Sequences were then classified against the Bacterial SILVA SEED database which excluded 21 sequences of chloroplasts. A distance matrix was calculated and sequences were clustered above a 97% similarity level which resulted in 2,137 OTUs grouping 133,197 reads. To reduce file complexity and to remove artefacts and contaminations, OTUs with less than 0.5% of the sequencing reads in a sample were excluded from the analysis. The latter cut-off was based on a mock community in which OTUs with less than 0.5% of the sequencing reads mostly contained only one or two reads and were assumed to be the result of sequencing errors. This procedure resulted in 28 OTUs representing 90.8% of the 133,197 reads. The taxonomic assignment of the selected OTUs was performed through EzTaxon-e analysis of representative sequences [Kim et al., 2012].

### 3.1.4 Results

Microbial diversity of the bumblebee gut

A total of 1940 isolates was collected from gut samples of 60 bumblebees representing four *Bombus* species (Supplementary table 3.1). Their MALDI-TOF MS profiles grouped into 67 clusters which corresponded to 52 species as revealed by subsequent gene sequence analyses (Figure 3.1) [Kim et al., 2014]. Accession numbers for 16S rRNA gene sequences are LK054485-LK054489, LN713847, and LT631737-LT631782. Accession numbers for *pheS*, *hsp60* and *groEL* sequences are LM999917-LM999921 and LT631783-LT631803. Accession numbers for 26S rRNA gene sequences are LT631804-LT631809 and LT798898. Several species were represented by multiple MALDI-TOF MS clusters (Figure 3.1), indicating that strains with slightly different MALDI-TOF MS spectra were isolated for these species [Ghyselinck et al., 2011]. Most rarefaction curves of sampling locations and bumblebee species did not reach saturation (Supplementary figures 3.1 and 3.2).

Most isolated bacteria belonged to the Firmicutes and Actinobacteria phyla; yet also Alpha-, Beta-, and Gammaproteobacteria, Bacteroidetes and yeasts were isolated. The

most commonly isolated species were *Lactobacillus bombicola*, *Fructobacillus fructosus*, *Bombiscardovia coagulans*, *S. alvi*, *Fructobacillus tropaeoli* and *Gilliamella* spp. and the yeast species *Candida bombi* (Figure 3.1). A major fraction of the species isolated (n = 21) consisted of LAB and bifidobacteria.

To evaluate if all predominant taxa were isolated, 16S rRNA amplicon sequencing was performed on gut samples, pooled according to bumblebee species. The intra-sample rarefaction curves based on the normalised dataset with 0.5% cut-off reached saturation (Supplementary Figure 3.3). A total of 120,905 reads were obtained which grouped into 28 OTUs (Table 3.1). Most OTUs belonged to the Gammaproteobacteria (n= 11) and Firmicutes (n= 10). The remaining 7 OTUs belonged to the Alpha- and Betaproteobacteria, Bacteroidetes, Actinobacteria and Tenericutes phyla. OTUs identified as *Snodgrassella*, *Gilliamella* and *Bombiscardovia* contained the majority of the reads obtained for the *B. lapidarius* (60.77%) and *B. pascuorum* (86.92%) samples. In the *B. lapidarius* sample also an *Apibacter* and a *Pseudomonas* OTU represented considerable fractions of the reads (i.e. 12.55 and 5.98%, respectively). While the *Gilliamella* OTU was also dominant in the *B. lucorum* sample (35.49%), most reads in the latter sample corresponded to a *Lactococcus* (15.14%) and a *Hafnia* OTU (20.92%). The *B. terrestris* sample was dominated by a *Hafnia* and an *Enterobacter* OTU which comprised more than 70% of the reads.

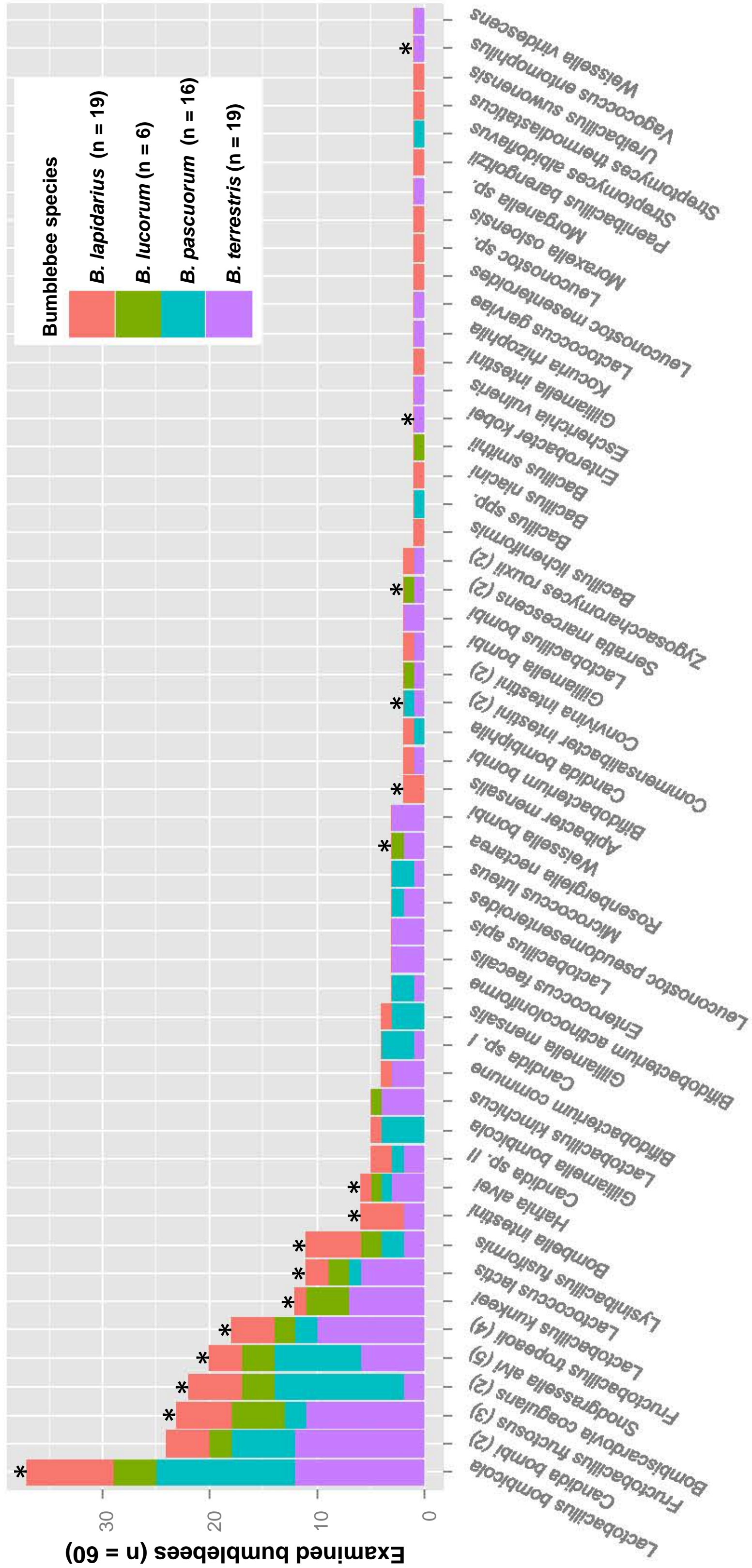



**Figure 3.1:** Prevalence of isolated microbial species per bumblebee species analyzed. Black stars indicate bacterial OTUs detected by 16S rRNA amplicon sequencing. If a species was represented by several MALDI-TOF MS clusters, the number of clusters is given next to the species name. Two distinct clusters of *Candida* sp. isolates were isolated and are presented as *Candida* sp. I and *Candida* sp. II.

**Table 3.1:** Relative abundance of the reads for each phylotype for the four analysed bumblebee species. Phylotypes of which isolates were obtained are indicated in bold character type.

| Phylum | Nearest neighbour[a] | Name OTU in literature[b] | *B. lapidarius* | *B. lucorum* | *B. pascuorum* | *B. terrestris* |
|---|---|---|---|---|---|---|
| Alphaproteobacteria | ***Bombella intestini*** 98.4% | **Alpha-2.2** | 1.81 | 5.41 | 0.07 | 4.34 |
| | ***Commensalibacter intestini*** 97.2% | | 0.05 | 3.75 | 0.17 | 0.89 |
| | *Phyllobacterium brassicacearum* 99.3% | | 0.77 | 0.00 | 0.02 | 0.00 |
| Betaproteobacteria | **Snodgrassella alvi** 98.5% | **Beta** | 23.69 | 2.15 | 33.85 | 1.18 |
| Gammaproteobacteria | ***Gilliamella mensalis*** and ***G. bombicola*** 98.7% | **Gamma-1** | 29.27 | 35.49 | 40.33 | 1.01 |
| | *Gilliamella apicola* 95.5% | | 5.95 | 0.00 | 0.00 | 0.00 |
| | *Schmidhempelia bombi* 98.5% | | 0.00 | 3.57 | 0.00 | 0.20 |
| | ***Hafnia alvei*** 99.1% | **Gamma-E2** | 0.87 | 20.92 | 0.87 | 29.60 |
| | ***Enterobacter hormaechei*** 99.1% | | 0.00 | 0.07 | 0.00 | 43.40 |
| | *Pseudomonas* spp. 92.6% | Gamma-II[c] | 5.98 | 0.48 | 0.00 | 0.24 |
| | ***Serratia marcescens*** 98.9% | | 0.00 | 2.04 | 0.00 | 0.00 |
| | ***Rosenbergiella nectarea*** 99.4% | | 0.15 | 1.11 | 0.01 | 0.75 |
| | *Buttiauxella agrestis* 99.1% | Gamma-E1 | 0.00 | 0.34 | 0.00 | 4.81 |
| | *Pantoea eucalypti* 97.8% | | 0.00 | 0.01 | 0.00 | 2.85 |
| | *Shimwellia blattae* 97.9% | | 0.00 | 0.00 | 0.00 | 2.98 |
| Bacteroidetes | ***Apibacter mensalis*** 99.4% | **Bacteroidetes** | 12.55 | 0.08 | 0.00 | 0.00 |

Continued on next page.

Continued from previous page.

| Phylum | Nearest neighbour[a] | Name OTU in literature[b] | *B. lapidarius* | *B. lucorum* | *B. pascuorum* | *B. terrestris* |
|---|---|---|---|---|---|---|
| Firmicutes | *Lactobacillus nodensis* 89.0% | Lacto-4 | 0.32 | 0.01 | 0.00 | 0.00 |
| | *Lactobacillus nodensis* 89.0% | Lacto-4 | 3.31 | 0.07 | 0.03 | 0.20 |
| | ***Lactobacillus bombicola*** 99.6% | **Lacto1-Firm-5** | 2.71 | 0.26 | 0.88 | 0.21 |
| | ***Lactobacillus kunkeei*** 99.8% | **Lacto-3** | 0.63 | 15.14 | 0.00 | 0.43 |
| | ***Fructobacillus tropeaoli*** 99.6% | | 1.60 | 2.51 | 0.97 | 0.66 |
| | ***Fructobacillus fructosus*** 99.1% | **Fructobacillus** | 0.02 | 1.06 | 0.00 | 0.02 |
| | ***Lactococcus lactis*** 99.1% | **Firm-S** | 0.00 | 4.35 | 0.00 | 1.60 |
| | ***Vagococcus entomophilus*** 99.8% | | 0.00 | 0.00 | 0.00 | 1.52 |
| | *Bacillus* spp. 99.8% | | 2.05 | 0.00 | 0.16 | 0.01 |
| | ***Lysinibacillus fusiformis*** 99.1% | | 0.00 | 0.00 | 0.00 | 2.20 |
| Actinobacteria | ***Bombiscardovia coagulans*** 98.2% | **Bifido-3** | 7.81 | 1.18 | 12.74 | 0.88 |
| Tenericutes | *Spiroplasma melliferum* 99.6% | | 0.74 | 0.00 | 0.00 | 0.00 |
| Total number of reads | | | 41368 | 35510 | 34976 | 9051 |

[a] Identity based on a 16S rRNA gene sequence of approximately 460 bp.

[b] The names of the phylotypes are those used by Meeus et al. [2015] unless stated otherwise.

[c] The name of the phylotype is that used by Koch and Schmid-Hempel [2011b].

Comparative analysis of the cultivated gut microbiota composition

The microbial community differences between the samples were visualized by a NMDS plot (Figure 3.2). Although the *B. lapidarius* and *B. lucorum* microbial community profiles and those of the sampling locations were more evenly distributed, the *B. pascuorum* and *B. terrestris* profiles grouped according to species. The stress value is a goodness of fit measure of the NMDS analysis which represents the difference between calculated distances and distances in the two-dimensional plot [Holland, 2008]. A low stress value (stress = 0.12) was obtained indicating that the distances can be accurately plotted in two dimensions. Based on the ANOSIM analysis, the community profiles shared a high degree of similarity ($R < 0.5$). However, significant differences were observed between the microbial community profiles of bumblebee species ($R = 0.14$ and p-value $<0.001$) and sampling locations ($R = 0.12$ and p-value $<0.05$). No pairwise significant differences were observed for the sampling locations but the community profiles of the *B. pascuorum* samples differed significantly from those of the *B. terrestris* ($R = 0.33$ and p-value $<0.001$) and *B. lapidarius* samples ($R= 0.12$ and p-value $<0.05$). The highest degree of separation was observed between the community profiles of *B. terrestris* and *B. pascuorum* ($R = 0.33$). SIMPER analysis revealed an average Bray-Curtis dissimilarity of 78.39% and 77.46% for *B. terrestris* and *B. pascuorum* and for *B. lapidarius* and *B. pascuorum*, respectively. The microbial species which together contributed to more than 50% of this dissimilarity are displayed in Supplementary Table 3.2. The observed differences were due to a higher frequency of *B. coagulans*, *S. alvi* and *L. bombicola* in *B. pascuorum* compared to *B. terrestris* and *B. lapidarius.* A higher frequency of *C. bombi*, *F. fructosus*, *F. tropeaoli*, *Lactococcus lactis* and *Lactobacillus kunkeei* was observed in *B. terrestris* compared to *B. pascuorum.* The frequency of *C. bombi* and *Gilliamella bombicola* was lower in *B. lapidarius* compared to *B. pascuorum*, while a higher frequency of *Lysinibacillus fusiformis* was observed in *B. lapidarius.*

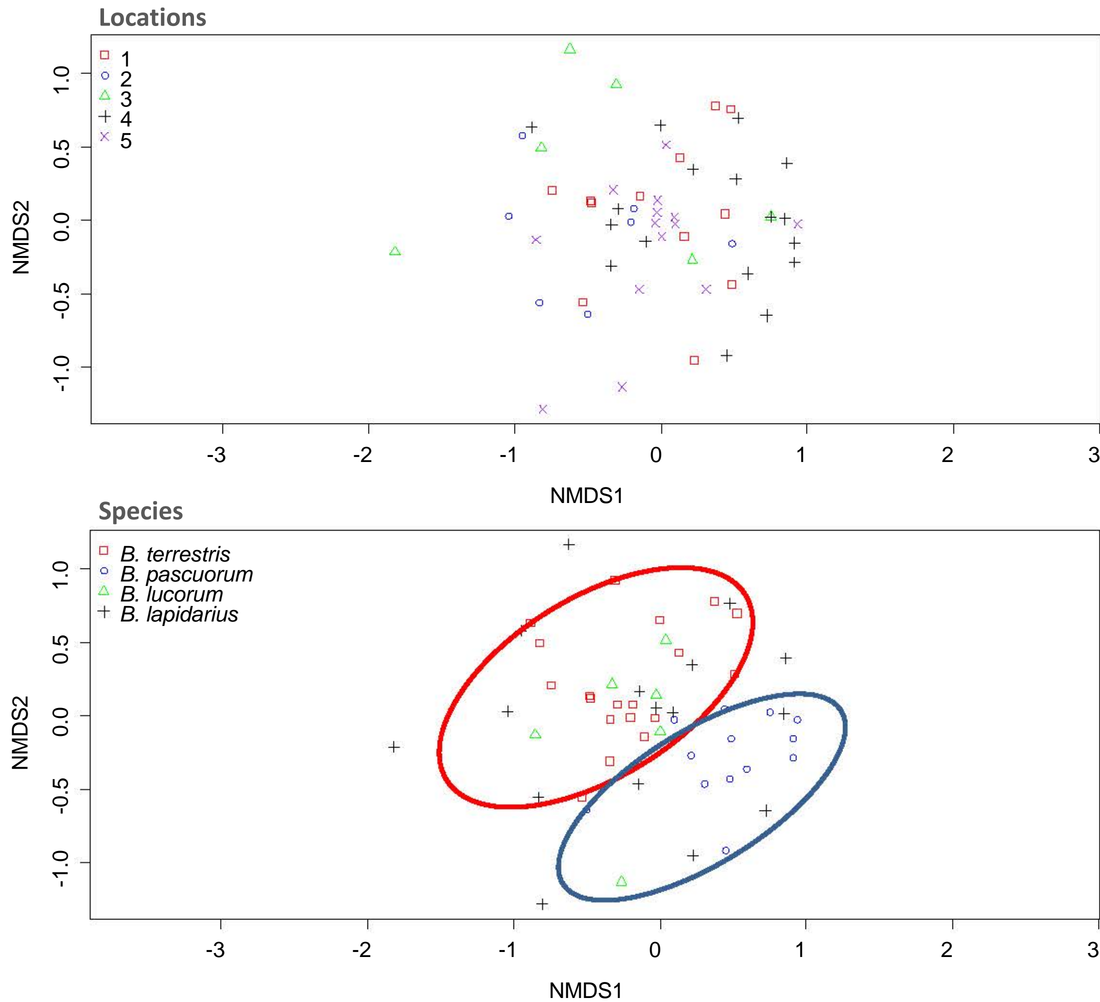



**Figure 3.2:** NMDS plot of microbial profiles of bumblebee gut samples. The red and blue circles indicate the distinct grouping of the *B. terrestris* and *B. pascuorum* profiles. The bumblebee species and sampling locations are indicated with different symbols and colours. Location 1 = Bourgoyen Ghent, location 2 = Coupure Ghent, location 3 = Gentbrugge, location 4 = Ledeganck botanical garden Ghent, location 5 = Den blakken botanical garden Wetteren.

Pathogen inhibition assays

Several of the 233 tested isolates inhibited growth of *P. larvae* and *M. plutonius* strains *in vitro* (Table 3.2). This inhibitory effect was strain rather than species specific (Table 3.2). When the assay was performed with cell-free supernatant instead of cell suspensions, growth was no longer inhibited. Only *Commensalibacter intestini* R-53529 inhibited growth of *A. apis*. Cell-free supernatant of the isolate again did not inhibit growth. Pathogen growth was not inhibited when AC broth adjusted to pH 3 was added to the wells. The addition of 0.1 M or 1 M lactic acid or acetic acid in AC broth to the plates inoculated with the *P. larvae* and *M. plutonius* strains resulted in inhibition zones of about 3 mm for 0.1 M lactic or acetic acid and 1 cm for 1 M lactic or acetic acid. *A. apis* growth was inhibited by the addition of 1 M acetic acid (inhibition zone of about 5 mm).

Of the 233 tested isolates, six inhibited growth of *C. bombi in vitro* (Figure 3.3), i.e. each *W. bombi* [n = 4], *Weissella viridescens* [n = 1] and *S. albidoflavus* [n = 1] isolate tested. The supernatant of these isolates was subsequently adjusted to pH 5.8 or to pH 7 with 1 M NaOH. Additionally, supernatant adjusted to pH 7 was treated with catalase, proteinase K or trypsin. The growth inhibitory activity of the supernatant of the five *Weissella* isolates was blocked when catalase was added. Neither of the above mentioned approaches eliminated the inhibitory activity of the supernatant of the *Streptomyces* strain.

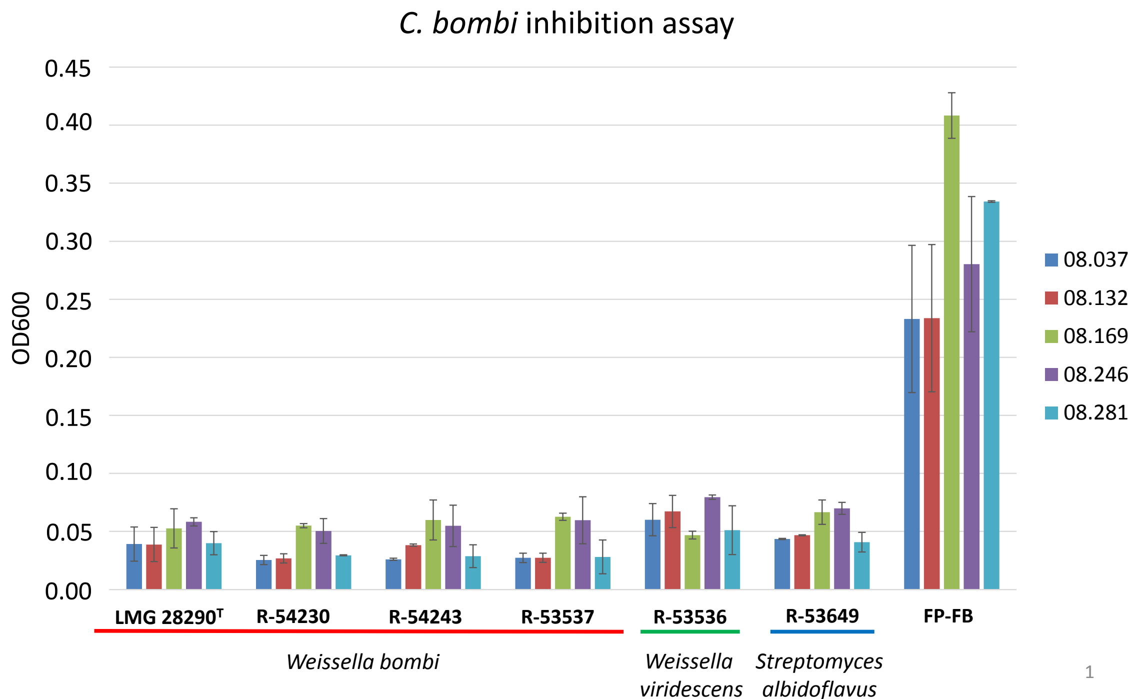



**Figure 3.3:** OD600 measurements for *Weissella bombi* LMG 28290$^T$, R-54230, R-54243 and R-53537, *Weissella viridescens* R-53536 and *Streptomyces albidoflavus* R-53649 inhibiting growth of five *C. bombi* strains (08.037, 08.132, 08.169, 08.246 and 08.281). FP-FB growth medium was used as a negative control.

**Table 3.2:** Number of bumblebee gut isolates with antimicrobial activity against foulbrood pathogens.

| Phylum | Species | No. of tested isolates | *P. larvae* LMG 9820$^T$ | *P. larvae* R-54592 | *P. larvae* R-54334 | *M. plutonius* LMG 20360$^T$ |
|---|---|---|---|---|---|---|
| Alphaproteobacteria | *Bombella intestini* | 1 | 1 | 1 | 1 | 0 |
| | *Commensalibacter intestini* | 1 | 1 | 1 | 1 | 0 |
| Betaproteobacteria | *Snodgrassella alvi* | 20 | 0 | 0 | 0 | 0 |
| Gammaproteobacteria | *Enterobacter kobei* | 1 | 0 | 0 | 0 | 0 |
| | *Gilliamella bombi* | 1 | 0 | 0 | 0 | 0 |
| | *Gilliamella bombicola* | 2 | 0 | 0 | 0 | 0 |
| | *Gilliamella intestini* | 1 | 0 | 0 | 0 | 0 |
| | *Gilliamella mensalis* | 1 | 0 | 0 | 0 | 0 |
| | *Hafnia alvei* | 8 | 5 | 5 | 3 | 1 |
| | *Moraxella osloensis* | 1 | 0 | 0 | 0 | 0 |
| | *Rosenbergiella nectareae* | 1 | 0 | 0 | 0 | 0 |
| | *Serratia marcescens* | 2 | 2 | 2 | 2 | 1 |
| Bacteroidetes | *Apibacter mensalis* | 2 | 0 | 0 | 0 | 0 |
| Firmicutes | *Bacillus licheniformis* | 1 | 0 | 0 | 0 | 0 |
| | *Bacillus* spp. | 1 | 0 | 0 | 0 | 0 |
| | *Convivina intestini* | 3 | 3 | 3 | 3 | 2 |
| | *Enterococcus faecalis* | 3 | 3 | 3 | 3 | 3 |
| | *Fructobacillus fructosus* | 24 | 18 | 18 | 18 | 0 |
| | *Fructobacillus tropeaoli* | 16 | 13 | 13 | 13 | 0 |
| | *Lactobacillus apis* | 2 | 2 | 2 | 2 | 2 |
| | *Lactobacillus bombi* | 2 | 0 | 0 | 0 | 0 |
| | *Lactobacillus bombicola* | 38 | 17 | 9 | 11 | 6 |
| | *Lactobacillus kimchicus* | 5 | 3 | 3 | 3 | 2 |
| | *Lactobacillus kunkeei* | 13 | 7 | 8 | 9 | 1 |
| | *Lactococcus lactis* | 10 | 6 | 6 | 6 | 2 |
| | *Leuconostoc mesenteroides* | 1 | 1 | 1 | 1 | 0 |
| | *Leuconostoc pseudomesenteroides* | 3 | 3 | 3 | 3 | 3 |
| | *Leuconostoc* sp. | 1 | 0 | 0 | 0 | 0 |
| | *Lysinibacillus fusiformis* | 8 | 0 | 0 | 0 | 0 |
| | *Paenibacillus barengoltzii* | 1 | 0 | 0 | 0 | 0 |
| | *Ureibacillus suwonensis* | 1 | 0 | 0 | 0 | 0 |
| | *Vagococcus entomophilus* | 1 | 1 | 1 | 1 | 1 |
| | *Weissella bombi* | 5 | 5 | 5 | 5 | 5 |
| | *Weissella viridescens* | 1 | 1 | 1 | 1 | 1 |
| Actinobacteria | *Bifidobacterium actinocoloniforme* | 2 | 0 | 0 | 0 | 0 |
| | *Bifidobacterium bombi* | 2 | 2 | 2 | 2 | 0 |
| | *Bifidobacterium commune* | 5 | 0 | 0 | 0 | 0 |
| | *Bombiscardovia coagulans* | 21 | 0 | 0 | 0 | 0 |
| | *Kocuria rhizophila* | 2 | 0 | 0 | 0 | 0 |
| | *Micrococcus luteus* | 1 | 0 | 0 | 0 | 0 |
| | *Streptomyces albidoflavus* | 1 | 0 | 0 | 0 | 0 |
| Fungi | *Candida* sp. I | 4 | 0 | 0 | 0 | 0 |
| | *Candida* sp. II | 1 | 0 | 0 | 0 | 0 |
| | *Candida bombi* | 8 | 0 | 0 | 0 | 0 |
| | *Candida bombiphila* | 2 | 0 | 0 | 0 | 0 |
| | *Zygosaccharomyces rouxii* | 2 | 0 | 0 | 0 | 0 |
| | | 233 | 94 | 87 | 88 | 30 |

Pectin degradation assay

Only a single bumblebee isolate, i.e. *B. licheniformis* R-53713, was able to degrade pectin and PGA. A halo (+/- 8 mm) was visible on MP-7 medium with pectin at pH 7 and on MP-7 medium with PGA at pH 5 (Supplementary figure 3.4).

Genome sequence analysis

No secondary metabolites with potential antimicrobial activity were detected in the genome of *W. bombi* LMG 28290$^{T}$ but two enzymes commonly involved in hydrogen peroxide production (pyruvate oxidase and NADH-flavin reductase) were present (Supplementary table 3.3). Twenty-three secondary metabolite gene clusters were detected in the genome of the *S. albidoflavus* isolate (R-53649) corresponding to non-ribosomal peptide, polyketide, terpene, lantipeptide and bacteriocin synthesis (Supplementary table 3.4). A complete pathway for pectin degradation was observed in the genome of *B. licheniformis* R-53713 (Supplementary table 3.3).

### 3.1.5 Discussion

Diversity of the bumblebee gut microbiota

In the present study, we carried out a comprehensive isolation campaign of the bumblebee gut microbiota to generate a more complete and accurate picture of its bacterial species diversity. A single general and rich medium (AC agar) was used along with selective media for the isolation of LAB (MRS agar) and bifidobacteria (MTPY and M144 agar). Isolation media were incubated at 37°C in different atmospheres and yielded 1940 isolates representing 52 different species (Supplementary table 3.1 and Figure 3.1). Rarefaction curves per bumblebee species or per sampling area generally did not reach saturation, indicating that analysis of an increasing number of samples would have revealed an even higher species diversity (Supplementary figures 3.1 and 3.2).

Isolates were obtained for all consistently reported bumblebee phylotypes i.e. *L. bombicola* and *L. apis* which correspond to the Firm-5/Lacto-1 phylotype, and *Gilliamella* spp., *S. alvi* and *B. coagulans* which correspond to the Gamma-1, Beta and Bifido-3 phylotypes, respectively [Meeus et al., 2015; Martinson et al., 2011; Koch and Schmid-Hempel, 2011b]. Except for Lacto-4, isolates were also obtained for all more inconsistently reported phylotypes, i.e. *Lactobacillus bombi* (Firm-4/Lacto-2), *L. kunkeei* (Lacto-3), *Lactobacillus kimchicus* (Lacto-5), *L. lactis* (Firm-S), *F. fructosus* (Fructobacillus), *Bifidobacterium commune* (Bifido-X), *Bifidobacterium actinocoloniiforme* (Bifido-1), Bifidobacterium bombi (Bifido-2), *Bombella intestini* (Alpha-2.2), *Hafnia alvei* (Gamma-E2), *Serratia marcescens* (Gamma) and *Apibacter mensalis* (Bacteroidetes) [Meeus et al., 2015; Martinson et al., 2011; Koch and Schmid-Hempel, 2011b]. Most of the remaining species that were isolated are associated with environmental sources, like for instance *Rosenbergiella nectarea* which has been isolated from nectar, or *Streptomyces* spp., *Bacillus* spp., *Micrococcus luteus* and *Kocuria rhizophila* which have been isolated from soil [Halpern et al., 2013]. While no yeast specific isolation media were used and the single general isolation medium comprised 10 ppm of cycloheximide, also several yeast species were isolated with *C. bombi* as the most prevalent one. Data presented in Figure 3.1 indicate that yeast species can be quite prevalent in the bumblebee gut and that a proper evaluation of the yeast diversity and of their functional role is required.

Our data demonstrated that the bacterial species diversity of the bumblebee gut is underestimated. Several phylotypes detected through 16S rRNA amplicon sequencing consisted of multiple species: four novel *Gilliamella* species (Gamma-1), *A. mensalis* (Bacteroidetes) and *L. bombicola* (Firm-5/Lacto-1) were first isolated in the course of the present study [Praet et al., 2016; Li et al., 2014a; Praet et al., 2017]. In addition, several lactic acid bacteria, i.e. *W. bombi*, *F. tropeaoli*, *Convivina intestini* and *Leuconostoc* spp., are reported for the first time in the bumblebee gut.

Pairwise significant differences were observed between the cultivated gut microbiota communities of *B. pascuorum* and *B. terrestris* and of *B. pascuorum* and *B. lapidarius* while this was not observed between those of other pairs of bumblebee species or between sampling locations (Figure 3.2). This corresponds to the observations of Koch and Schmid-Hempel [2011b] and Cariveau et al. [2014] which found differences between bumblebee species based on cultivation independent studies and might be explained by a different host ecology or physiology. *B. pascuorum* was characterized by a higher frequency of *B. coagulans*, *S. alvi* and *L. bombicola* which are species belonging to consistently reported phylotypes while *B. terrestris* and *B. lapidarius* were characterized by a higher frequency of species belonging to more erratically detected phylotypes like *F. fructosus* and *L. lactis*. In addition, the prevalence of species belonging to the *Enterobacteriaceae* was higher in *B. lucorum* and *B. terrestris* samples while in the *B. terrestris* sample more than 70% of the reads consisted of a *Hafnia* and *Enterobacter* phylotype. Clearly, *Enterobacteriaceae* can also be dominant members of the bumblebee gut. This conforms with the observation of Li et al. [2015] that two enterotypes exist in bumblebees: one dominated by *Gilliamella* and *Snodgrassella* and one dominated by *Enterobacteriaceae*.

As mentioned above, most rarefaction analyses failed to reach saturation (Supplementary figures 3.1 and 3.2). To further evaluate the success of the isolation campaign, we performed 16S rRNA amplicon sequencing on all gut samples, pooled by bumblebee species. This demonstrated that isolates were obtained for most of the detected phylotypes (Table 3.1), except for 11 low abundant phylotypes, most of which represent environmental organisms. Two of these low abundant phylotypes, i.e., Gamma-II and Lacto-4, are commonly detected in the bumblebee gut [Koch and Schmid-Hempel, 2011b; Meeus et al., 2015]. As is the case for Candidatus *Schmidhempelia bombi* [Martinson et al., 2014], all present cultivation efforts have failed to yield isolates that would facilitate a functional characterization of these bacteria. The 16S rRNA gene sequences of the Gamma-II and Lacto-4 phylotypes showed at most 92.6% and 89.0% sequence identity, respectively, to those of established species, indicating that these bacteria too represent novel genera, specific to their bumblebee host.

Functionality of the bumblebee gut microbiota

Although the gut microbiota is increasingly recognized as important to bee health [Zheng et al., 2016; Engel et al., 2012; Koch and Schmid-Hempel, 2011a], the functional potential of most of these micro-organisms remains elusive. We selected 233 isolates to test their capacity

to mediate pectin degradation and to inhibit pathogen growth *in vitro*.

In honeybees, live LAB are able to protect larvae against infection with the American and European foulbrood pathogens, *P. larvae* and *M. plutonius* [Vasquez et al., 2012; Forsgren et al., 2009; Audisio et al., 2011; Killer et al., 2013]. Bumblebees probably encounter these pathogens through shared flower use with honeybees, however, no disease symptoms have been reported. In the present study, a range of LAB isolates was able to inhibit growth of these pathogens *in vitro* (Table 3.2). This inhibitory effect was lost when cell-free supernatant was used. The same effect was observed for *L. apis* LMG 26964$^{T}$ which was used as a positive control. Growth inhibition of *P. larvae* and *M. plutonius* has previously been reported for the latter strain but the inhibition mechanism was not further investigated [Killer et al., 2013]. Pathogen growth was not inhibited by AC broth adjusted to pH 3, indicating that the observed inhibition was likely not caused by a pH effect. The inhibitory effect may be explained by nutrient competition, by a higher concentration of antimicrobial compounds when isolates grow in agar wells compared to broth medium, or by the production of antimicrobials which are not stable in liquid medium [Mahenthiralingam et al., 2011; Le Breton et al., 2007]. We demonstrated that *P. larvae* and *M. plutonius* are sensitive to 0.1 M lactic and acetic acid. Therefore, the production of short chain fatty acids by isolates growing in the wells might inhibit pathogen growth *in vivo* as well.

We also investigated the potential of the isolates to inhibit growth of the fungal pathogen *A. apis* which occurs in multiple bee species and causes chalkbrood disease in honeybees [Evison et al., 2012; Graystock et al., 2016b]. Vegetative and reproductive stages of this pathogen have recently been detected in diseased bumblebee queens raised in captivity [Maxfield-Taylor et al., 2015]. Of the 233 isolates examined, only *Commensalibacter intestini* R-53529 inhibited growth of *A. apis* MUCL 34668. Also, this inhibition was lost when supernatant was used and was again not likely caused by a pH effect as AC broth adjusted to pH 3 did not inhibit growth. Growth inhibition might be caused by the production of acetic acid by *C. intestini* R-53529, as growth of *A. apis* could be inhibited by addition of 1 M acetic acid to the well diffusion assay.

*C. bombi* is a bumblebee-specific trypanosomatid gut parasite associated with slow colony growth and reduced colony founding [Shykoff and Schmid-hempel, 1991; Yourth et al., 2008]. The supernatant of only six isolates (all *W. bombi* [n = 4], *W. viridescens* [n = 1] and *S. albidoflavus* [n = 1] isolates) inhibited growth of the *C. bombi* strains analyzed (Figure 3.3). Possible pH effects were ruled out by adjusting the pH of the supernatant to pH 5.8 and pH 7. Supernatant adjusted to pH 7 was treated with catalase, proteinase K or trypsin. Neither of these treatments could eliminate the observed growth inhibition caused by *S. albidoflavus* R-53649, excluding therefore proteins or hydrogen peroxide as mechanisms of action. Several antimicrobial compounds produced by *S. albidoflavus* have been described, i.e. a sesquiterpene albaflavenone, a polyketide antimycin A18 and a straight-chain polyhydroxy, polyether, non-proteinaceous compound with antifungal activity [Augustine et al., 2005; Guertler et al., 1994; Yan et al., 2010]. Analysis of the genome sequence of *S. albidoflavus*

R-53649 revealed the presence of several biosynthetic gene clusters for secondary metabolites with a potential antimicrobial activity (Supplementary table 3.4). Further studies have to be performed to identify the active compound. Growth inhibition caused by the *Weissella* isolates was reversed by the addition of catalase and is therefore likely caused by the production of hydrogen peroxide.

Of the 233 tested isolates, only *B. licheniformis* R-53713 was able to degrade pectin and polygalacturonic acid *in vitro* (Supplementary figure 3.4), and a complete pathway for pectin degradation was present in its genome (Supplementary table 3.3). Indeed, *B. licheniformis* is known to produce pectate lyases, pectin lyases and polygalacturonases [Rehman et al., 2012; Remoroza et al., 2015]. *B. licheniformis* R-53713 exclusively degraded pectin at pH 7 and PGA at pH 5 which might be explained by a different pH optimum of the enzymes involved [Rehman et al., 2012; Remoroza et al., 2015]. The ability to degrade PGA has been shown for several *Gilliamella* strains [Zheng et al., 2016; Engel et al., 2012], however was not observed for the *Gilliamella* isolates in the present study. Genome sequence analysis revealed that one *Gilliamella* isolate (LMG 28359$^{T}$) contains a complete pathway for pectate degradation while the genes for galacturonic acid degradation were detected in the genome of another *Gilliamella* isolate (LMG 29880$^{T}$) [Praet et al., 2017]. Unsuitable experimental conditions might explain why pectate degradation was not detected *in vitro* for these isolates or that the interaction of gut microbionts rather than individual strains may be responsible for the PGA degradation reported in previous studies [Zheng et al., 2016; Engel et al., 2012].

In conclusion, in the present study we collected 1940 isolates from gut samples of 60 bumblebees representing four *Bombus* species and demonstrated that the bacterial species diversity in these *Bombus* species exceeded that suggested by phylotype analysis through 16S rRNA amplicon sequencing considerably. The composition of the cultivable gut microbiota of *B. pascuorum* and of *B. terrestris* was unique; that of *B. lapidarius* and *B. lucorum* could not be distinguished, yet of the latter only six bees were analysed. While representatives of most phylotypes reported earlier and detected in the present study were effectively isolated, the isolation of several low abundant phylotypes like Candidatus *Schmidhempelia bombi*, Gamma-II, Lacto-4 and Gamma-E1, remained elusive. Although most rarefaction analyses failed to reach saturation we speculate that this was caused by the sporadic isolation of environmental bacteria. Finally, our data demonstrated that the prevalence and diversity of yeasts in the bumblebee gut may be underestimated. We also demonstrated that several of the obtained isolates inhibit growth of the bee pathogens *P. larvae*, *M. plutonius*, *A. apis* and *C. bombi in vitro*, but the mechanisms of action require further studies. Finally, only one in 233 isolates tested was able to degrade pectin and polygalacturonic acid *in vitro*.

### 3.1.6 Acknowledgements

The authors acknowledge support by Ghent University-Special Research Fund (BOF).

### 3.1.7 Conflict of interest

The present research involved sampling of bumblebees for which no permission was required as bumblebees are not included in the "Decree of Species (het Soortenbesluit (http://codex.vlaanderen.be/Zoeken/Document.aspx?DID=1018227¶m=informatie])" of the Flemish government with inception on 01/09/2009. The authors do not have a conflict of interest.
The genome sequence data of LMG 28290$^{T}$ was produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) in collaboration with the user community. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

### 3.1.8 Supplementary material

**Supplementary Table 3.1:** Isolation media, growth atmosphere, and number of isolates per microbial species. If multiple MALDI-TOF MS clusters were observed for a species, the number of MALDI-TOF MS clusters is given between brackets.

| Phylum | Species | AC M | M144 AN | MRS A | MRS AN | MTPY AN | Total |
|---|---|---|---|---|---|---|---|
| **Alphaproteobacteria** | *Bombella intestini* | 21 | | | | | 21 |
| | *Commensalibacter intestini* (2) | 28 | | | | | 28 |
| **Betaproteobacteria** | *Snodgrassella alvi* (5) | 102 | | | 4 | 4 | 110 |
| **Gammaproteobacteria** | *Enterobacter kobei* | 2 | | | | | 2 |
| | *Gilliamella bombi* | 2 | | | 1 | | 3 |
| | *Gilliamella bombicola* | 22 | | | | | 22 |
| | *Gilliamella intestini* | | 4 | | | | 4 |
| | *Gilliamella mensalis* | 5 | 8 | | | | 13 |
| | *Hafnia alvei* | 63 | 6 | 29 | 25 | | 123 |
| | *Escherichia vulneris* | 2 | | | | | 2 |
| | *Moraxella osloensis* | 2 | | | | | 2 |
| | *Morganella morganii* | 3 | | | | | 3 |
| | *Rosenbergiella nectareae* | 10 | | | | | 10 |
| | *Serratia marcescens* (2) | 4 | | | | | 4 |
| **Bacteroidetes** | *Apibacter mensalis* | 2 | | | | | 2 |
| **Firmicutes** | *Bacillus licheniformis* | | | | | 1 | 1 |
| | *Bacillus niacini* | 1 | | | | | 1 |
| | *Bacillus smithii* | 1 | | | | | 1 |
| | *Bacillus* sp. | 2 | | | | | 2 |
| | *Convivina intestini* (2) | 17 | | 8 | 8 | 1 | 34 |
| | *Enterococcus faecalis* | 17 | | 6 | 4 | 12 | 39 |
| | *Fructobacillus fructosus* (3) | 66 | 3 | 23 | 51 | 8 | 151 |
| | *Fructobacillus tropeaoli* (4) | 115 | | 118 | 82 | 13 | 328 |
| | *Lactobacillus apis* | | | | 2 | 12 | 14 |
| | *Lactobacillus bombi* | | | | 2 | 1 | 3 |
| | *Lactobacillus bombicola* | 4 | 9 | 10 | 127 | 90 | 240 |
| | *Lactobacillus kimchicus* | 17 | | 15 | 7 | | 39 |
| | *Lactobacillus kunkeei* | 31 | | 84 | 4 | 2 | 121 |
| | *Lactococcus garviae* | 1 | | | 3 | 3 | 7 |
| | *Lactococcus lactis* | 102 | | 11 | 57 | 25 | 195 |
| | *Leuconostoc mesenteroides* | | | 10 | | | 10 |
| | *Leuconostoc pseudomesenteroides* | 18 | 1 | | | | 19 |
| | *Leuconostoc* sp. | | | | 1 | | 1 |
| | *Lysinibacillus fusiformis* | 16 | | 1 | | | 17 |
| | *Paenibacillus barengoltzii* | 1 | | | | | 1 |
| | *Ureibacillus suwonensis* | 1 | | | | | 1 |
| | *Vagococcus entomophilus* | 1 | | | | | 1 |
| | *Weissella bombi* | 11 | | 3 | 8 | 1 | 23 |
| | *Weissella viridescens* | 6 | | 3 | | | 9 |
| **Actinobacteria** | *Bifidobacterium actinocoloniforme* | | | | | 5 | 5 |
| | *Bifidobacterium bombi* | | | | 1 | 6 | 7 |
| | *Bifidobacterium commune* | 4 | | | 2 | 11 | 17 |
| | *Bombiscardovia coagulans* (2) | | | | 2 | 115 | 117 |
| | *Kocuria rhizophila* | 4 | | | | | 4 |
| | *Micrococcus luteus* | 14 | | | | | 14 |
| | *Streptomyces albidoflavus* | 1 | | | | | 1 |
| | *Streptomyces thermodiastaticus* | | 1 | | | | 1 |
| **Fungi** | *Candida bombi* (2) | 31 | 4 | 108 | 1 | | 144 |
| | *Candida* sp. I | | | 7 | | | 7 |
| | *Candida bombiphila* | | | 4 | | | 4 |
| | *Candida* sp. II | 1 | | 7 | | | 8 |
| | *Zygosaccharomyces rouxii* (2) | 1 | | 3 | | | 4 |
| | **Total** | 752 | 36 | 450 | 392 | 310 | 1940 |

A = aerobic, AN = anaerobic and M = micro-aerobic atmosphere

**Supplementary Table 3.2:** SIMPER analysis showing species ranked according to their contribution to the Bray-Curtis dissimilarity between *B. terrestris* and *B. pascuorum* and between *B. lapidarius* and *B. pascuorum.* The list of species was limited to a cumulative percentage dissimilarity of 50%.

| Species | Frequency[a] *B. terrestris* | Frequency *B. pascuorum* | Contrib.%[b] | Cum. %[c] |
|---|---|---|---|---|
| *Bombiscardovia coagulans* | 0.105 | 0.75 | 9.846 | 9.846 |
| *Candida bombi* | 0.579 | 0.313 | 7.273 | 17.12 |
| *Fructobacillus fructosus* | 0.579 | 0.125 | 6.909 | 24.03 |
| *Fructobacillus tropeaoli* | 0.526 | 0.125 | 6.678 | 30.71 |
| *Snodgrassella alvi* | 0.316 | 0.5 | 6.453 | 37.16 |
| *Lactobacillus bombicola* | 0.632 | 0.813 | 5.783 | 42.94 |
| *Lactococcus lactis* | 0.316 | 0.0625 | 4.96 | 47.9 |
| *Lactobacillus kunkeei* | 0.368 | 0 | 4.639 | 52.54 |
| Species | Frequency *B. terrestris* | Frequency *B. pascuorum* | Contrib.% | Cum. % |
| *Bombiscardovia coagulans* | 0.294 | 0.75 | 12.89 | 12.89 |
| *Lactobacillus bombicola* | 0.471 | 0.813 | 10.33 | 23.22 |
| *Snodgrassella alvi* | 0.176 | 0.5 | 8.463 | 31.68 |
| *Candida bombi* | 0.235 | 0.313 | 6.297 | 37.98 |
| *Gilliamella bombicola* | 0.0588 | 0.313 | 6.145 | 44.13 |
| *Lysinibacillus fusiformis* | 0.294 | 0.125 | 6.055 | 50.18 |

[a]Frequency of microbial species in the respective bumblebee species.

[b]Percentage of contribution to the dissimilarity.

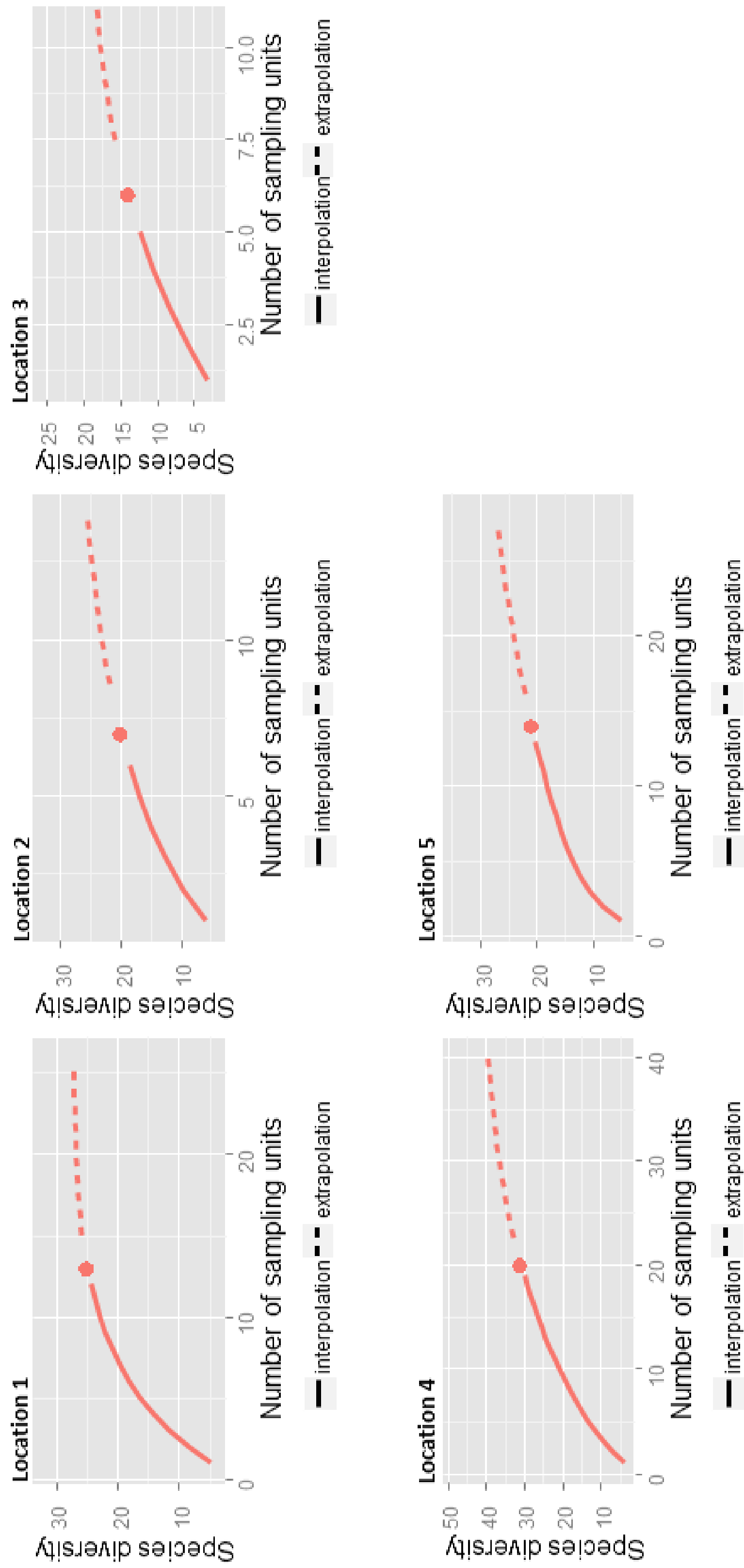
[c]Cumulative percentage of contribution to the dissimilarity.

**Supplementary Table 3.3:** Genome sequence analysis of *B. licheniformis* R-53713 and *W. bombi* LMG 28290$^T$.

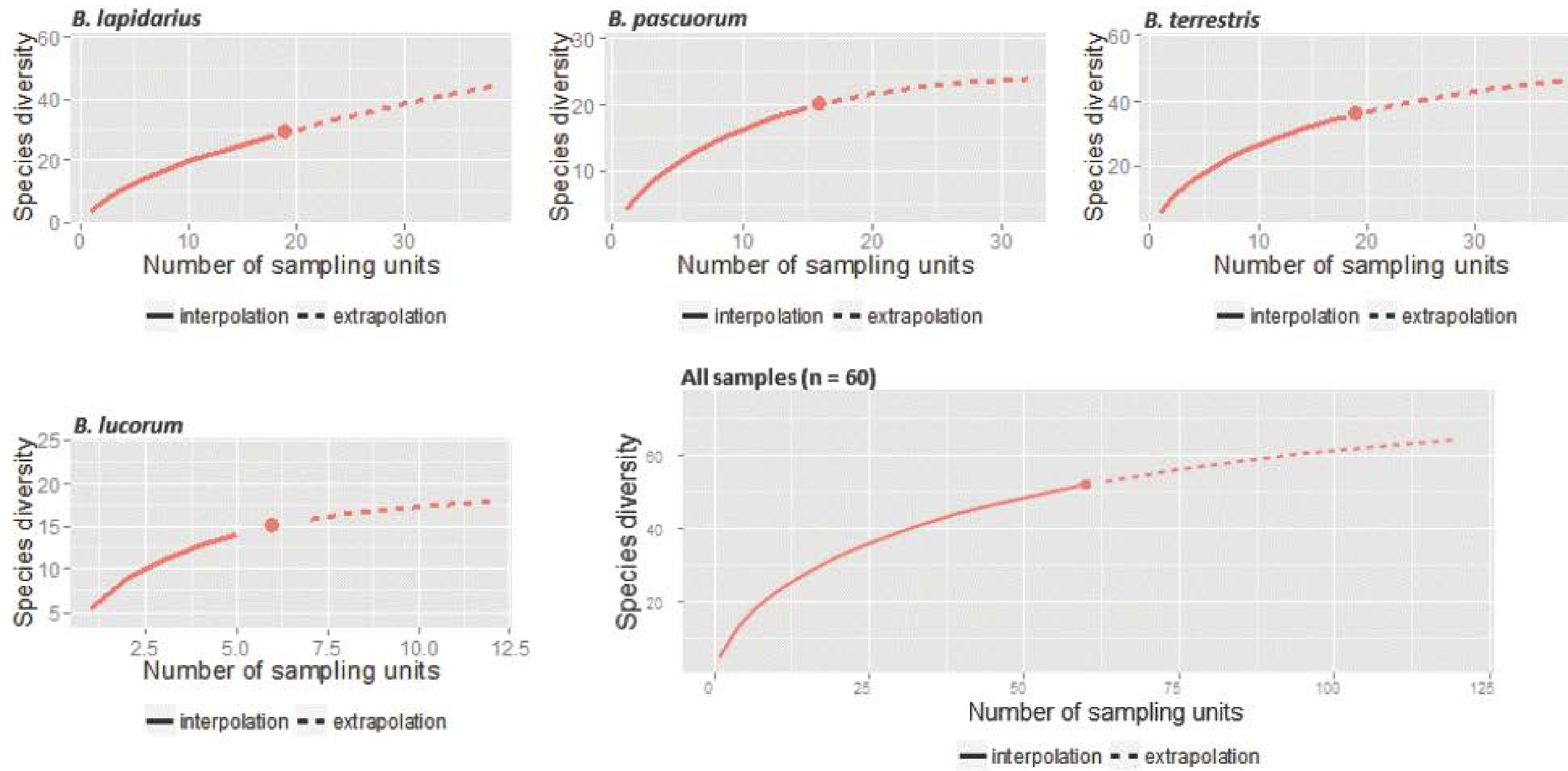
| | Protein | Function | scaffold | start | stop |
|---|---|---|---|---|---|
| ***B. licheniformis* R-53713** | ExuR | transcriptional repressor | 4 | 157422 | 156424 |
| **(Pectin degradation)** | ExuT | hexuronate transporter | 4 | 324464 | 323181 |
| | | | 6 | 389182 | 390453 |
| | PelA (EC 4.2.2.2) | pectate lyase | 4 | 750694 | 751719 |
| | | (Pectate to 4-(4-Deoxy-α-D-gluc-4-enuronosyl)-D-galacturonate and pectate) | 6 | 441882 | 443162 |
| | PelB (EC 3.2.1.15) | endo-polygalacturonase | 4 | 153390 | 152080 |
| | | (Polygalacturonic acid to digalacturonate or D-galacturonate) | | | |
| | UxaC (EC 5.3.1.12) | uronate isomerase | 4 | 16047 | 158926 |
| | | (galacturonate to tagaturonate) | | | |
| | UxaB (EC 1.1.1.58) | Altronate oxidoreductase | 4 | 156363 | 154894 |
| | | (tagaturonate to altronate) | | | |
| | UxaA (EC 4.2.1.7) | Altronate dehydratase | 4 | 154894 | 153404 |
| | | (altronate to 2-dehydro-3-deoxy-D-gluconate (KDG)) | | | |
| | KdgT | KDG permease | 4 | 451561 | 450575 |
| | KdgK (EC 2.7.1.45) | Kdg kinase | 4 | 453231 | 452266 |
| | | (KDG to 6-phopho-2-keto-3-deoxygluconate) | | | |
| | | | 4 | 695119 | 694193 |
| | KdgA (EC 4.1.3.16/ EC 4.1.2.14) | aldolase | 4 | 452260 | 451646 |
| | | (6-phopho-2-keto-3-deoxygluconate | | | |
| | | to pyruvate and 3-phosphoglyceraldehyde) | | | |
| ***W. bombi* LMG 28290$^T$** | Pox (EC 1.2.3.3) | pyruvate oxidase | 15 | 18574 | 20394 |
| **(hydrogen peroxide production)** | | NADH-flavin reductase | 1 | 267538 | 266882 |

**Supplementary Table 3.4:** Secondary metabolite biosynthesis gene clusters identified in the genome of *S. albidoflavus* R-53649.

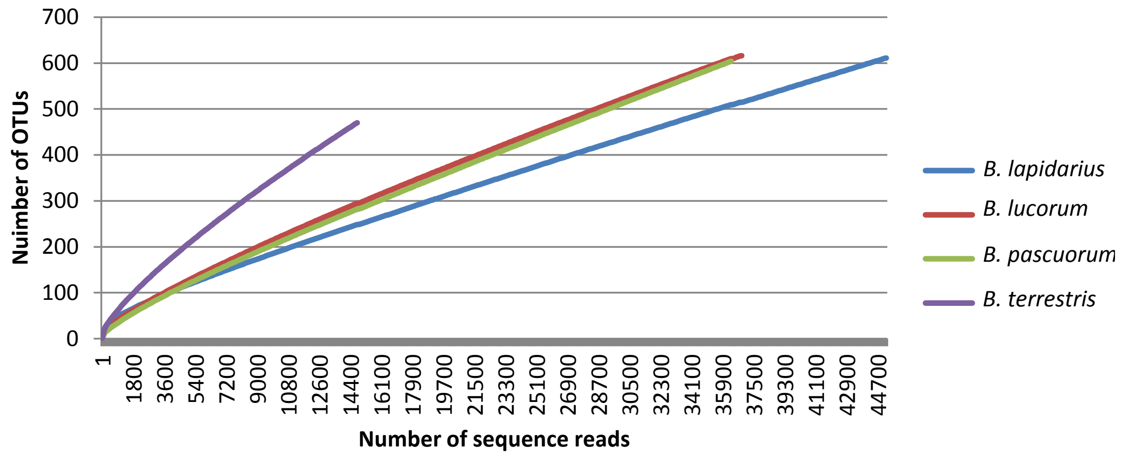
| Type of secondary metabolite biosynthesis gene cluster | Contig (start-stop) |
|---|---|
| **non-ribosomal peptides** | 50 (148-37941) |
| | 56 (1-50617) |
| | 68 (1846-42812) |
| | 73 (1-35048) |
| | 102 (1-25986) |
| | 179 (1-20825) |
| **Terpene** | 58 (38145-58523) |
| | 76 (2629-31516) |
| | 186 (565-24196) |
| **Type I PKS** | 3 (1-59547) |
| | 4 (46438-121860) |
| | 208 (1-12010) |
| **Type I PKS-NRPS** | 25 (5609-61353) |
| | 33 (73552-122962) |
| **Type III PKS** | 4 (388-41485) |
| **Bacteriocin-terpene** | 51 (16763-41689) |
| **Bacteriocin (TOMM microcin)** | 75 (1027-2979) |
| | 109 (28820-24426) |
| **Bacteriocin** | 160 (5524-22506) |
| | 262 (2384-8679) |
| **Lantipeptide** | 9 (23013-48061) |
| | 24 (1-3717) |
| | 64 (1-8567) |

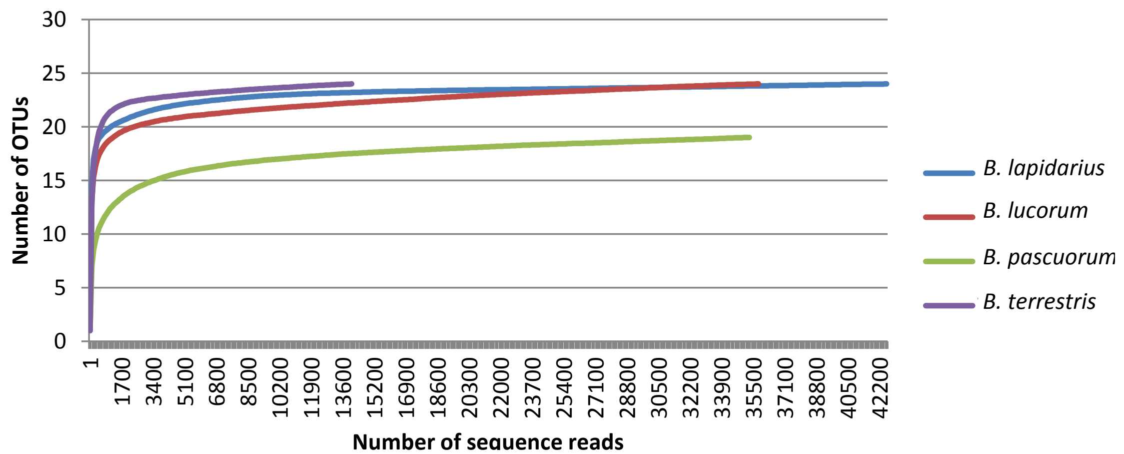



**Supplementary Figure 3.1:** Rarefaction curves of the sampled locations.

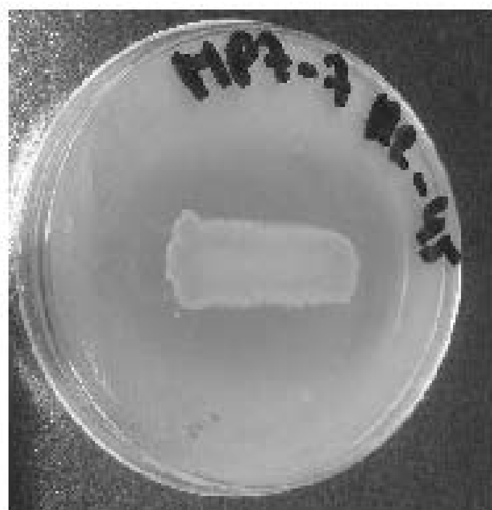



**Supplementary Figure 3.2:** Rarefaction curves of the sampled bumblebee species and the total collection of samples.




**Supplementary Figure 3.3:** Intra-sample rarefaction curves based on the normalised 16S rRNA amplicon sequencing datasets.



**Supplementary Figure 3.4:** Pectin degradation assay (MP-7 medium pH 7) of *Bacillus licheniformis* R-53713.

# CHAPTER 4

## Taxonomy of the bumblebee gut microbiota

During the course of the isolation study, several isolates could not be assigned to an existing microbial species and were formally described by the polyphasic taxonomic approach. Most of these novel species belong to bacterial phylotypes previously detected in the bumblebee gut. Section 4.1 includes the description of *Bombella intestini* which corresponds to the Alpha 2.2 phylotype. Section 4.2 and 4.3 describe novel LAB and bifidobacteria of which *Lactobacillus bombicola* and *Bifidobacterium commune* correspond to the Firm–5 and Bifido-X phylotype, respectively. Section 4.2 also includes the description of *Convivina intestini* and *Weissella bombi*, two novel LAB species which have previously not been detected in the bumblebee gut. *Apibacter mensalis* and four *Gilliamella* species are formally described in Section 4.4 and Section 4.5 and correspond to the Bacteroidetes and Gamma-1 phylotypes, respectively.

## 4.1 *Bombella intestini* gen. nov., sp. nov., an acetic acid bacterium isolated from bumblebee crop

**Redrafted from:** Leilei Li, Jessy Praet, Wim Borremans, Olga C. Nunes, Célia M. Manaia, Ilse Cleenwerck, Ivan Meeus, Guy Smagghe, Luc De Vuyst and Peter Vandamme (2015). *Bombella intestini* gen. nov., sp. nov., an acetic acid bacterium isolated from bumblebee crop. International Journal of Systematic and Evolutionary Microbiology, 65, 267–273

**Author contributions:** LL and PV conceived the study. LL, JP and PV wrote the manuscript. LL, WB, ON and CM performed the experiments and data-analysis. JP performed the sampling and dissection of the bumblebees. JP, IC, IM, GS and LDV proofread the manuscript.

### 4.1.1 Abstract

In the frame of a bumblebee gut microbiota study, acetic acid bacteria (AAB) were isolated using a combination of direct isolation methods and enrichment procedures. MALDI–TOF MS profiling of the isolates and a comparison of these profiles with profiles of established AAB species identified most isolates as *Asaia astilbes* or as '*Commensalibacter intestini*', except for two isolates (R–52486 and LMG 28161$^T$) that showed an identical profile. A nearly complete 16S rRNA gene sequence of strain LMG 28161$^T$ was determined and showed the highest pairwise similarity to *Saccharibacter floricola* S–877$^T$ (96.5%), which corresponded with genus level divergence in the family *Acetobacteraceae*. Isolate LMG 28161$^T$ was subjected to whole–genome shotgun sequencing; a 16S–23S rRNA internal transcribed spacer (ITS) sequence as well as partial sequences of the housekeeping genes *dnaK*, *groEL* and *rpoB* were extracted for phylogenetic analyses. The obtained data confirmed that this isolate is best classified into a new genus in the family *Acetobacteraceae*. The DNA G+C content of strain LMG 28161$^T$ was 54.9 mol%. The fatty acid compositions of isolates R–52486 and LMG 28161$^T$ were similar to those of established AAB species [with $C_{18:1}\omega7c$ (43.1%) as the major component], but the amounts of fatty acids such as $C_{19:0}$cyclo$\omega$8c, $C_{14:0}$ and $C_{14:0}$ 2–OH enabled to differentiate them. The major ubiquinone was Q–10. Both isolates could also be differentiated from the known genera of AAB by means of biochemical characteristics, such as their inability to oxidize ethanol to acetic acid, negligible acid production from melibiose, and notable acid production from D–fructose, sucrose and D–mannitol. In addition, they produced 2–keto–D–gluconate, but not 5–keto–D–gluconate from D–glucose. Therefore, the name *Bombella intestini* gen nov., sp. nov. is proposed for this new taxon, with LMG 28161$^T$ (DSM 28636$^T$, R–52487$^T$) as the type strain of the type species.

### 4.1.2 Introduction

Recently, acetic acid bacteria (AAB) have been isolated from various sources, mainly sugar–rich or alcoholic niches, such as vinegar, wine and flowers [De Vuyst et al., 2008; Kersters et al., 2006; Muthukumarasamy et al., 2005]. Some AAB species have symbiotic relationships with insects [Crotti et al., 2010]. In particular, species of the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, *Asaia* and *Saccharibacter* have frequently been associated with honeybees (*Apis mellifera*) [Babendreier et al., 2007; Crotti et al., 2010; Martinson et al., 2011; Mohr and Tebbe, 2006; Crotti et al., 2009; Gilliam, 1997], but only rarely with bumblebees (*Bombus* spp.) [Koch and Schmid-Hempel, 2011b; Martinson et al., 2011; Olofsson and Vasquez, 2009]. Yet, a recent study has revealed two *Acetobacteraceae* as relevant bacteria of the *Bombus bimaculatus* gut microbiota [Cariveau et al., 2014].

### 4.1.3 Methods, Results and Discussion

In the frame of a bumblebee gut microbiota study, bumblebees were caught in the field, in Ghent, Belgium, frozen at –20°C for 10 min and surface–sterilized with 2.5% Umonium[38] Master (Laboratoire Huckert's International) before dissecting their honey crop and whole gut. The crops and guts were homogenized in 500 µl saline solution [Olofsson and Vásquez, 2008] with a sterile micro–pestle and stored at –20°C until further use. Isolation of AAB from these suspensions was carried out using a combination of direct isolation methods and enrichment procedures. For the latter, 50 µl suspension was added to 25 ml of both enrichment medium I (EM I) and II (EM II), as described by Lisdiyanti et al. [2002], except that the pH was not adjusted; the final pH was therefore 6.8±0.2. The inoculated media were shaken at 100 rpm and incubated at 28°C for 7 days. Enrichment media showing bacterial growth were subsequently plated onto four solid agar media: LMG medium 13 [2.5% (w/v) D–mannitol; 0.5% (w/v) yeast extract (Oxoid); 0.3% (w/v) bacteriological peptone (Oxoid) and 1.5% (w/v) agar]; LMG medium 404 [5% (w/v) D–glucose; 1% (w/v) yeast extract (Oxoid) and 1.5% (w/v) agar]; LMG medium 405 [5% (w/v) D–glucose; 1% (w/v) yeast extract (Oxoid); 2% (v/v) ethanol; 1% (v/v) acetic acid and 1.5% (w/v) agar] and modified deoxycholate–mannitol–sorbitol (mDMS) medium [1% (w/v) bacteriological peptone (Oxoid); 0.3% (w/v) yeast extract (Oxoid); 0.63% (w/v) lactic acid; 0.5% (w/v) ethanol; 0.1% (w/v) D–glucose; 0.1% (w/v) sorbitol; 0.1% (w/v) mannitol; 0.1% (w/v) potassium hydrogen phosphate; 0.01% (w/v) sodium deoxycholate; 0.002% (w/v) magnesium sulphate heptahydrate; 0.003% (w/v) bromocresol purple and 1.8% (w/v) agar; pH 4.5] [Papalexandratou et al., 2013]. Each of the four media contained 100 ppm cycloheximide to inhibit yeast growth. For direct isolation, the cell suspensions were serially diluted to $10^{-7}$ in physiological water [0.85% (w/v) NaCl], and 50 µl of each dilution was plated directly onto the four solid agar media mentioned above. All inoculated plates were incubated aerobically at 28 °C for 7 days. LMG media 13 and 404 were the sole media that yielded growth. Colonies were picked from agar media that were inoculated both directly as well as after enrichment in EM II. Isolates (Supplementary Table 4.1) were dereplicated by matrix–assisted laser desorption/ionization

time–of–flight mass spectrometry (MALDI–TOF MS), as described by Wieme et al. [2012]. Cluster analysis of the MALDI–TOF MS profiles of these isolates revealed eight groups, of which two could be identified as *Asaia astilbes* and '*Commensalibacter intestini*' after comparing their profiles with profiles of established AAB species (Supplementary Figure 4.1). The other six groups could not be identified, and representative isolates were selected for 16S rRNA gene sequence analysis using the method described previously [Snauwaert et al., 2013]. NCBI BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the 16S rRNA gene sequences obtained (1353–1431 nt) revealed that all isolates, except for one, namely LMG 28161$^T$ (=R–52487$^T$), were lactic acid bacteria (data not shown). The 16S rRNA gene sequence of isolate LMG 28161$^T$ (1450 nt) showed 99% similarity to several uncultured *Acetobacteraceae* from the honeybee gut, crop or larvae [Koch et al., 2013; Martinson et al., 2011; Anderson et al., 2013; Mohr and Tebbe, 2007], which indicated that this strain was a member of the family *Acetobacteraceae*. Isolate LMG 28161$^T$ formed a single MALDI–TOF MS cluster with isolate R–52486, which showed an identical mass spectrum (Figure 4.2) and which was isolated from the same bumblebee crop sample. The 16S rRNA gene sequence of LMG 28161$^T$ was then compared with 16S rRNA gene sequences of type strains of all AAB species retrieved from the EMBL and NCBI databases, using the BioNumerics version 5.1 software (Applied Maths, Sint–Martens–Latem, Belgium). Isolate LMG 28161$^T$ showed the highest pairwise 16S rRNA gene sequence similarity to *Saccharibacter floricola* S–877$^T$ (96.5%; GenBank accession number AB110421). This rather low nearest neighbour similarity value suggested that the novel isolate should be classified into a new genus of the family *Acetobacteraceae*. The 16S rRNA gene sequences of isolate LMG 28161$^T$ and type strains of species representing all known genera of this family were aligned against the SILVA bacteria database using the Mothur pipeline [Schloss et al., 2009; Quast et al., 2013]. Subsequently, phylogenetic trees were reconstructed with the MEGA6 software package, using the neighbour–joining and maximum–likelihood methods [Felsenstein, 1981; Saitou and Nei, 1987]. The DNA substitution TN93+G+I was selected under the Bayesian Information Criterion [Nei and Kumar, 2000; Tamura et al., 2013a]. The robustness of the topology of both trees was estimated by bootstrap analysis with 1000 replicates [Felsenstein, 1985]. Both trees showed highly similar topologies, and therefore only the maximum–likelihood tree is shown (Figure 4.1).

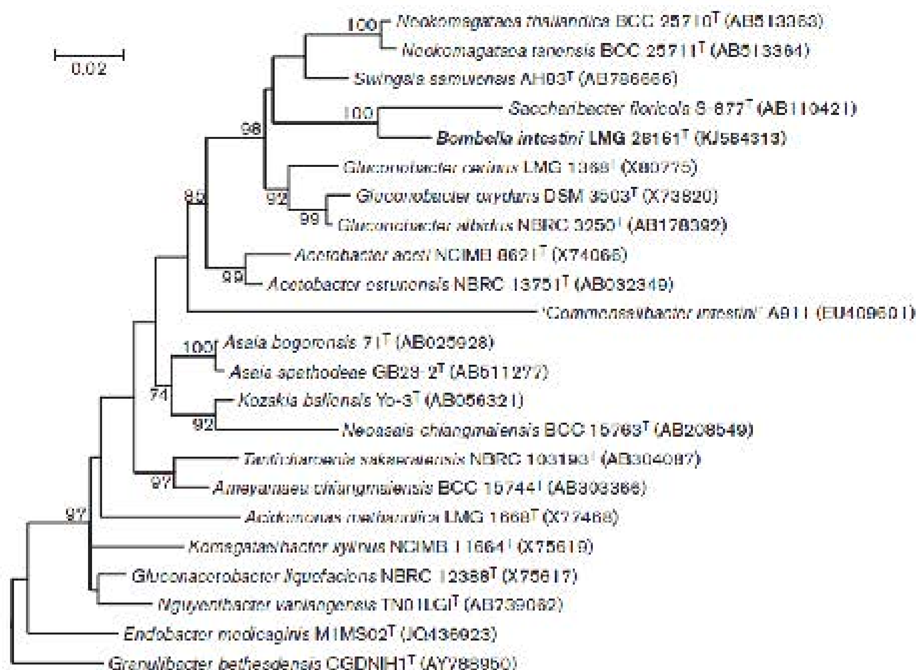



**Figure 4.1:** Maximum–likelihood tree based on nearly complete 16S rRNA gene sequences (1353–1431 nt), showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. within the family *Acetobacteraceae*. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 2% sequence divergence.

Genomic DNA of isolate LMG 28161$^T$ was obtained, as described previously [Cleenwerck et al., 2002], and subjected to whole–genome shotgun sequencing [Li et al., 2016]. A 16S–23S rRNA internal transcribed spacer (ITS) sequence and sequences of the housekeeping gene *groEL* were extracted for phylogenetic analyses, as these sequences were proven useful to refine the phylogeny of members of the family *Acetobacteraceae* [Cleenwerck et al., 2010; Li et al., 2014b; Trcek and Teuber, 2002]. A *groEL* sequence was also extracted from the draft genome of *S. floricola* DSM 15669$^T$ available online (GenBank accession number NZ_ARJS00000000.1). A phylogenetic analysis based on 16S–23S rRNA ITS sequences was performed using MEGA 6; a phylogenetic tree was reconstructed using the neighbour–joining method. The robustness of the tree topology was estimated by bootstrap analysis with 1000 replicates. The 16S–23S rRNA ITS analysis confirmed that isolate LMG 28161$^T$ could clearly be differentiated from its close neighbours (Supplementary Figure 4.3). The phylogenetic position of isolate LMG 28161$^T$, based on partial sequences of the housekeeping gene *groEL* (720 nt, (Supplementary Figure 4.4)), and based on the corresponding amino acid sequences (240 aa, (Supplementary Figure 4.5)), was also determined as described previously [Li et al., 2014b] using the MEGA 6 software. Again, isolate LMG 28161$^T$ formed a very distinct branch and the branch lengths obtained in the various trees further confirmed that isolate LMG 28161$^T$ should be best classified into a new genus of the family *Acetobacteraceae*.

Random amplified polymorphic DNA (RAPD) analysis was performed on isolates LMG $28161^T$ and R–52486, as described previously [Williams et al., 1990]. Two primers (RAPD–270 and RAPD–272) were used, which both yielded indistinguishable profiles, suggesting that LMG $28161^T$ and R–52486 are reisolates of the same strain (Supplementary Figure 4.6). The DNA G+C content of isolate LMG $28161^T$ was determined using the method described by Cleenwerck et al. [2008] and was 54.9 mol%. This value falls within the range reported for the family *Acetobacteraceae* [Greenberg et al., 2006; Sievers and Swings, 2005], and is also consistent with the whole–genome shotgun sequencing data, which revealed a value of 55.0 mol%.

The whole–cell fatty acid methyl esters (FAME) composition was determined for isolate LMG $28161^T$ and for the type strains of the type species of nearly all known genera of the family *Acetobacteraceae*, using an Agilent Technologies 6890N gas chromatograph. Cultivation of the strains and extraction of the FAME were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI), except that fatty acids were extracted from cultures grown on LMG medium 404 at 28°C under aerobic conditions for 48 to 72 h (depending on the strain). The peaks of the profiles were identified using the TSBA50 identification library version 5.0 (MIDI). The predominant fatty acid of strain LMG $28161^T$ was $C_{18:1}\omega7c$ (43.1%), while the following fatty acids were present in lesser amounts (>1%): $C_{19:0}$cyclo$\omega$8c (16.7%), $C_{16:0}$ (12.7%), $C_{14:0}$2–OH (8.5%), $C_{14:0}$ (9.3%), $C_{16:0}$2–OH (1.1%) and $C_{16:0}$3–OH (2.8%) (Table 4.1). The fatty acid composition of strain LMG $28161^T$ was similar to that of known AAB species (with $C_{18:1}\omega7c$ as the major component), but the amounts of fatty acids, such as $C_{19:0}$cyclo$\omega$8c, $C_{14:0}$ and $C_{14:0}$2–OH, enabled to differentiate it (Table 4.1 and Supplementary Table 4.2). The analysis of respiratory quinones of isolate LMG $28161^T$ was performed, as described previously [Vaz-Moreira et al., 2007] using the method of Tindall [1989]. The major ubiquinone present was Q–10, which agreed with the major ubiquinone of most other genera in the family *Acetobacteraceae*, except for species of the genus *Acetobacter* that have Q–9 as the major ubiquinone; no other quinones were found.

**Table 4.1:** Cellular fatty acids contents (%) of isolate LMG 28161$^{T}$ and the type strains of *Saccharibacter floricola* and *Gluconobacter oxydans*.

Strains: 1, *Bombella intestini* gen. nov., sp. nov. LMG 28161$^{T}$; 2, *Saccharibacter floricola* LMG 23170$^{T}$; 3, *Gluconobacter oxydans* LMG 1408$^{T}$. All data were generated in the frame of this study. Cultivation conditions prior to fatty acid extraction were identical for all strains. –, Not detectable or trace amount (<1%).

| **Fatty acid** | 1 | 2 | 3 |
|---|---|---|---|
| $C_{18:1}\omega 7c$ | 43.1 | 29.8 | 51 |
| $C_{19:0}$cyclo$\omega 8c$ | 16.7 | 28.4 | – |
| $C_{16:0}$ | 12.7 | 24.4 | 9.6 |
| $C_{14:0}$ | 9.3 | 2.8 | – |
| $C_{14:0}$ 2–OH | 8.5 | – | – |
| $C_{16:0}$ 3–OH | 2.8 | 2.9 | 6.7 |
| $C_{16:0}$ 2–OH | 1.1 | 6.4 | 20.8 |
| $C_{18:0}$ | – | 1.1 | 2.1 |
| $C_{18:0}$ 3–OH | – | – | 4.3 |

Both isolates LMG 28161$^{T}$ and R–52486 were subjected to phenotypic tests, using methods described previously [Cleenwerck et al., 2002, 2007]. For microscopy and colony morphology, isolates were grown aerobically at 28°C on LMG medium 404 for 48 h. On this medium, both isolates also grew when micro–aerobic conditions (80% $N_2$, 4%$O_2$, 8% $H_2$ and 8% $CO_2$) were used. As AAB are well known as obligate aerobic organisms, two close relatives (*S. floricola* LMG 23170$^{T}$ and *Gluconobacter oxydans* LMG 1408$^{T}$) were also tested for growth on LMG medium 404 after incubation at 28°C under micro–aerobic conditions. Both strains showed good growth, which seems logical, as species from these genera have been detected in the bee gut, which is a micro–aerobic environment. The biochemical characteristics examined for isolates LMG 28161$^{T}$ and R–52486 included: analysis of oxidase and catalase activity; oxidation of acetate and lactate; production of acetic acid from ethanol; growth in the presence of 30% (w/v) D–glucose; growth on methanol as carbon source; growth at 37°C on LMG medium 404; and assimilation of ammonium nitrogen on Frateur–Hoyer and Frateur modified Hoyer medium with D–glucose, D–mannitol and ethanol. The production of 2–keto–D–gluconate and 5–keto–D–gluconate from D–glucose was determined as reported previously [Spitaels et al., 2014]. In addition, acid production from various carbon sources was tested in triplicate, using the methods described previously [Asai et al., 1964]. *S. floricola* LMG 23170$^{T}$ was investigated concurrently for all the tests conducted. *Acetobacter aceti* LMG 1504$^{T}$ and *Gluconobacter roseus* LMG 1418$^{T}$ were included as controls for part of the acid production tests. Isolates R–52486 and LMG 28161$^{T}$ could be differentiated from genera of the family *Acetobacteraceae* by means of multiple biochemical characteristics (Table 4.2), such as their inability to produce acetic acid and 5–keto–D–gluconate from ethanol and D–glucose, respectively; their ability to grow at 37°C on LMG medium 404; their negligible acid production from melibiose; and notable acid production from D–fructose, sucrose and D–mannitol. For *S. floricola* LMG 23170$^{T}$, the phenotypic test results were not always congruent with published data, while for *Acetobacter aceti* LMG 1504$^{T}$ and

*Gluconobacter roseus* LMG 1418$^T$ the published data were confirmed (Table 4.2). Therefore, a nearly complete 16S rRNA gene sequence was determined for strain LMG 23170$^T$ (1436 nt, GenBank accession number KJ850435) and compared to publicly available 16S rRNA gene sequences of the type strain of *S. floricola*, including a sequence extracted from the draft genome. Pairwise sequence similarities of 100% with GenBank accession number JF794031 (1349 nt), 99.9% with a sequence extracted from the draft genome (NZ_KB899360, 1485 nt) and 99.8% with accession number AB110421 (1436 nt) were found, suggesting that strain LMG 23170$^T$ is a true subculture of the type strain of *S. floricola*.

In conclusion, the present study demonstrated that the taxon represented by the isolates R–52486 and LMG 28161$^T$ can be differentiated genotypically and phenotypically from established species and genera of the family *Acetobacteraceae*. The phylogenetic relationship between this taxon and its nearest neighbour species, *S. floricola*, is considerably divergent (Figure 4.1 and Supplementary Figures 4.3, 4.4 and 4.5) and its fatty acid profile is unique (Tables 4.1 and 4.2). Phenotypic characteristics that allow to distinguish this taxon from *S. floricola* are its ability to grow on LMG medium 404 at 37°C; its inability to oxidize lactate and produce 5–keto–D–gluconate from D–glucose; and its ability to produce acid from D–galactose, D–fructose, D–mannitol, L–arabinose and D–mannose (the latter two weakly) (Table 4.2). Therefore, the classification of this taxon as a novel species of a new genus of the family *Acetobacteraceae* is warranted. We propose the name *Bombella intestini* gen. nov., sp. nov., with strain LMG 28161$^T$ (=DSM 28636$^T$) as the type strain.

**Table 4.2:** Characteristics that differentiate *Bombella* gen. nov. from phylogenetically close relatives of the family *Acetobacteraceae*. Genera: 1, *Bombella* gen. nov. (data from this study); 2, *Saccharibacter* (this study and Jojima et al. [2004]); 3, *Gluconobacter* (this study and Lisdiyanti et al. [2002]); 4, *Swingsia* [Malimas et al., 2013]; 5, *Neokomagataea* [Yukphan et al., 2011]. All genera had Q–10 as the major ubiquinone. +, Positive; –, negative; W, weakly positive; V, variable; ND, not determined.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Motility and flagellation | non–motile | non–motile | polar or non–motile | non–motile | non–motile |
| Production of water–soluble | | | | | |
| brown pigment(s) | – | – | v | + | – |
| Growth on LMG medium 404 at 37°C | + | – | – | ND | ND |
| Oxidation of | | | | | |
| Acetate | – | –° | – | – | – |
| Lactate | – | w° | – | – | – |
| Production of acetic acid | | | | | |
| from ethanol | – | –* | + | w/+ | w |
| Growth in the presence of | | | | | |
| 30%(w/v) D–glucose | + | +° | – | + | + |
| Assimilation of ammoniacal nitrogen | | | | | |
| on Frateur–modified Hoyer medium with: | | | | | |
| D–Glucose | – | –* | + | – | ND |
| D–Mannitol | – | –* | + | + | ND |
| Ethanol | – | –* | – | – | ND |
| Production of keto–D–gluconates | | | | | |
| from D–glucose: | | | | | |
| 2–Keto–D–gluconate | + | +° | + | + | + |
| 5–Keto–D–gluconate | – | +° | + | + | + |
| Acid production from: | | | | | |
| L–Arabinose | w | –* | +° | w | v(+/w) |
| D–Arabinose | – | –° | + | w/– | – |
| D–Xylose | – | w* | +° | w | + |
| D–Glucose | + | w* | +° | + | + |
| D–Galactose | + | –* | +° | +/– | v(+/w) |
| D–Mannose | w | –* | +° | +/w | v(–/w) |
| D–Fructose | + | –* | + | w | + |
| L–Sorbose | – | –° | + | – | – |
| Melibiose | – | –* | +° | + | v |
| Sucrose | + | +° | + | +/w | + |
| Raffinose | – | –° | – | w | v |
| D–Mannitol | + | –* | +(w°) | + | – |
| D–Sorbitol | – | –° | + | –/w | – |
| Glycerol | – | –° | + | –/w | – |
| Ethanol | – | –° | + | – | v(w/–) |
| Major ubiquinone | Q–10 | Q–10 | Q–10 | Q–10 | Q–10 |
| DNA G+C content (mol%) | 54.8 | 52–53 | 52–64† | 46.9–47.3 | 51.2–56.8 |

°Data obtained in the present study.

* Data obtained in the present study not corresponding to published data.

†This data is taken from Cleenwerck and De Vos (2008).

### 4.1.4 Description of *Bombella* gen. nov.

*Bombella* (Bom.bel'la N.L. fem. dim. n. *Bombella*, named after the bumblebee genus, *Bombus*, from which the first isolate of this genus was obtained).

Cells are Gram–stain–negative, straight rods. Catalase activity is present, but oxidase activity is absent. The predominant fatty acid is $C_{18:1}\omega7c$; other fatty acids in significant amounts are $C_{19:0}$cyclo$\omega$8c, $C_{16:0}$, $C_{14:0}$2–OH, $C_{14:0}$, $C_{16:0}$2–OH and $C_{16:0}$3–OH. The major quinone type is Q–10. The type species is *Bombella intestini.* The presence of 16S rRNA gene fragments in the gut metagenomes of honeybees and bumblebees, which share 99% and 97% sequence similarity (query coverage of 34% and 48%, respectively) [Cariveau et al., 2014; Martinson et al., 2011] with the nearly complete 16S rRNA gene sequence of strain LMG $28161^{T}$ suggests that the occurrence of members of this taxon is not limited to bumblebees.

### 4.1.5 Description of *Bombella intestini* sp. nov.

*Bombella intestini* (in.tes.ti'ni. L. gen. n. *intestini* of the gut).

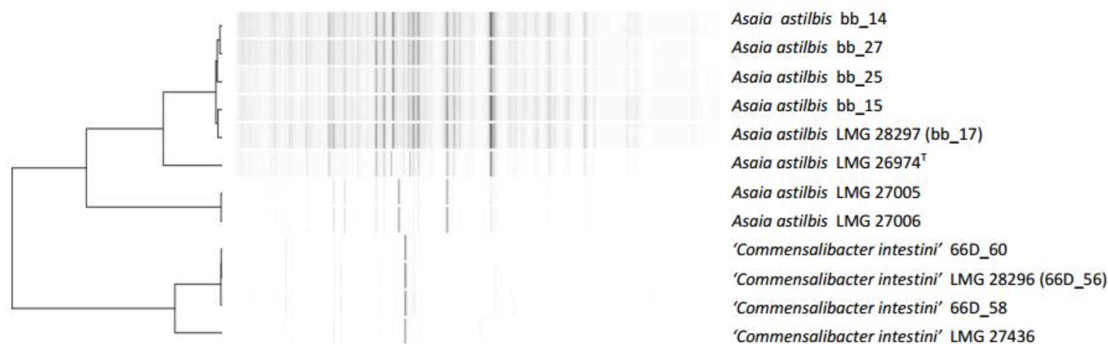
Characteristics are the same as those described for the genus with the following additional properties. Cells are non–motile, approximately 1 µm wide and 2–3 µm long. Cells occur singly or in pairs. On LMG medium 404 agar [5% (w/v) D–glucose; 1% (w/v) yeast extract (Oxoid) and 1.5% (w/v) agar], colonies are round, smooth, brownish and slightly raised, with a diameter of approximately 1–2 mm after 3 days incubation. Ethanol is not oxidized to acetic acid. Produces 2–keto–D–gluconate from D–glucose, but not 5–keto–D–gluconate. Produces acid from sucrose, D–fructose, D–glucose, D–mannitol, D–galactose, D–mannose (weakly) and L–arabinose (weakly). Grows in the presence of 30% (w/v) D–glucose. Growth is observed at 37°C on LMG medium 404. No growth is observed with ammonium as the sole nitrogen source.

The type strain, LMG $28161^{T}$ (=DSM $28636^{T}$ =R–$52487^{T}$), was isolated from the crop of a bumblebee (*Bombus lapidarius*) in 2013 in Ghent, Belgium. The DNA G+C content of the type strain is 54.9 mol%. An additional strain of the species is R–52486.

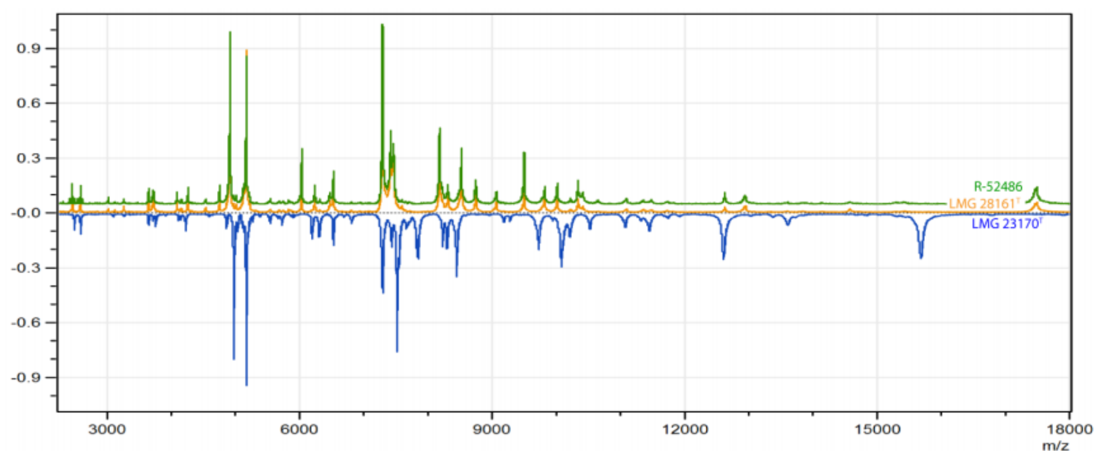
### 4.1.6 Acknowledgments

L. L. has a PhD grant from the Chinese Scholarship Council and Ghent University Co–Funding. The BCCM/LMG collection is supported by the Federal Public Planning Service –Science Policy, Belgium. P. V. and L. D. V. acknowledge their financial support of the University Research Council, the Research Foundation–Flanders (FWO–Vlaanderen), and the Hercules Foundation.

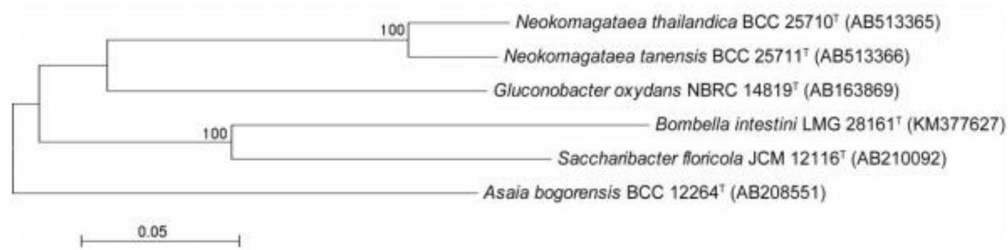
### 4.1.7 Supplementary material



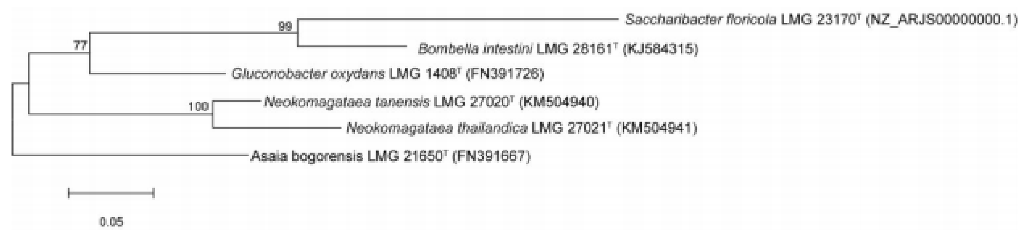

**Supplementary Figure 4.1:** MALDI–TOF MS identification of bumblebee isolates as *Asaia astilbes* and '*Commensalibacter intestini*'. LMG 26974$^{T}$, LMG 27005, LMG 27006 and LMG 27436 are reference strains. Isolates bb_17 and 66D_56 were deposited in the BCCM\LMG Bacteria Collection as LMG 28297 and LMG 28296, respectively.



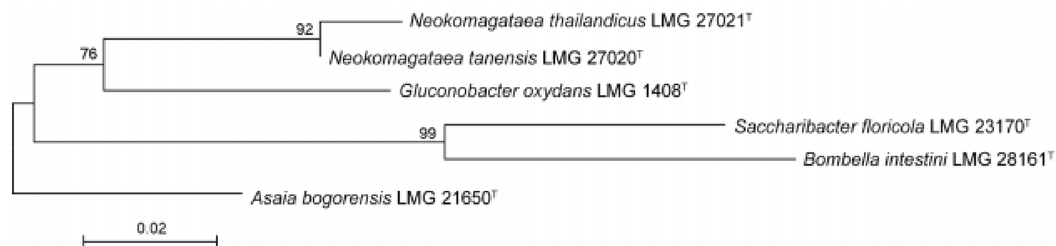

**Supplementary Figure 4.2:** Comparison of MALDI–TOF MS profiles of *Bombella intestini* gen. nov., sp. nov. LMG 28161$^{T}$ and R–52486, and *Saccharibacter floricola* LMG 23170$^{T}$ using the mMass 5.1.0 software [Strohalm et al., 2010]. The isolates LMG 28161$^{T}$ and R–52486 showed an identical mass spectrum.



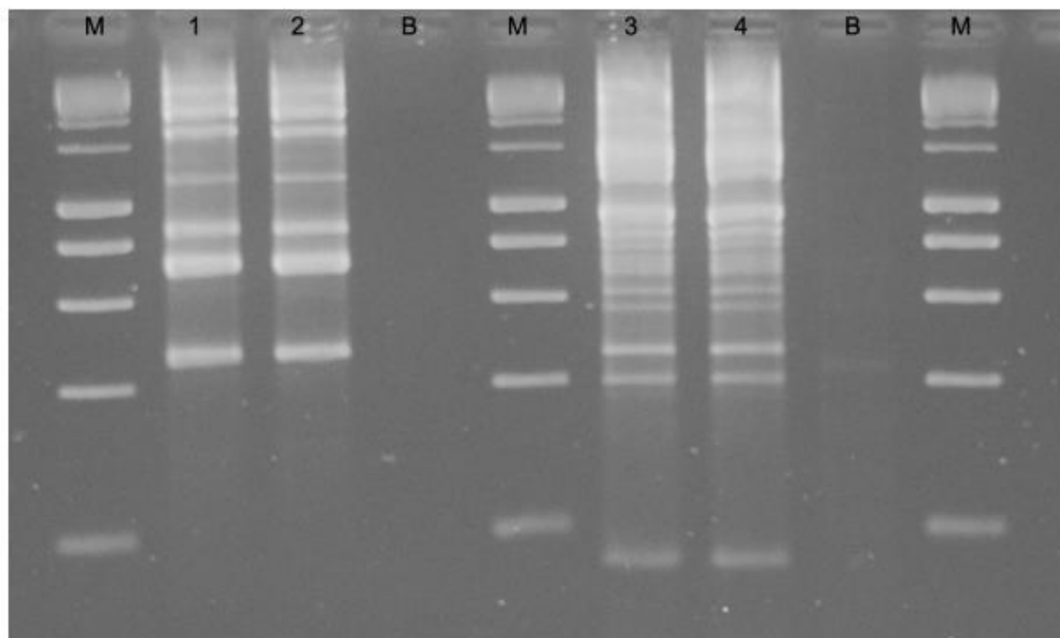

**Supplementary Figure 4.3:** Neighbour–joining tree based on 16S–23S rRNA gene internal transcribed spacer (ITS) sequences (560 nt of LMG 28161$^{T}$), showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. to its close relatives. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 5% sequence divergence.




**Supplementary Figure 4.4:** Maximum–likelihood tree based on partial housekeeping gene sequences *groEL* (720 nt), showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. to its close relatives. The nucleotide substitution model TN93+G was chosen. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 5% sequence divergence.




**Supplementary Figure 4.5:** Maximum–likelihood tree based on amino acid sequences of housekeeping gene *groEL* fragments (240 aa). The amino acid substitution model LG+G was chosen. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 2% sequence divergence.




**Supplementary Figure 4.6:** RAPD fingerprints of *Bombella intestini* gen. nov., sp. nov. R–52486 and LMG 28161$^{T}$. Lanes 1, 2 = RAPD patterns obtained using primer RAPD–270 (5'–TGCGCGCGGG–3') of R–52486 and LMG 28161$^{T}$, respectively. Lanes 3, 4 = RAPD patterns obtained using primer RAPD–272 (5'–AGCGGGCCAA–3') of R–52486 and LMG 28161$^{T}$, respectively. Lane M, reference marker. Lane B, blank.

**Supplementary Table 4.1:** Isolates from bumblebee gut samples and their identifications. (*Bombus* spp. were determined by colour pattern. *B. terrestris* and *B. lucorum* were similar in colour pattern thus could not be differentiated).

| Sample no. | Bumblebee species (*Bombus* spp.) | Sampling location | Isolates | Medium | Identification methods |
|---|---|---|---|---|---|
| 19 | *B. terrestris**lucorum* | Gentbrugge | *Asaia astilbes* | EM II + M13 | MALDI–TOF MS\\MLSA |
| 20 | *B. terrestris* *lucorum* | Gentbrugge | *Asaia astilbes* | EM II + M13 | MALDI–TOF MS\\MLSA |
| 22 | *B. lapidarius* | Gentbrugge | *Bombella intestini* | EM I + M13 | MALDI–TOF MS\\ nearly complete 16S rRNA gene sequencing\\MLSA |
| 21 | *B. terrestris**lucorum* | Gentbrugge | Closest to *Fructobacillus tropaeoli* sequence similarity 99% | EM II + M13 | nearly complete 16s rRNA gene sequencing (1518 bp) |
| 23 | *B. lapidarius* | Gentbrugge | Closest to *Fructobacillus tropaeoli* sequence similarity 99% | EM I + M13 | nearly complete 16s rRNA gene sequencing (1518 bp) |
| 24 | *B. terrestris*\\lucorum | Gentbrugge | Closest to *Fructobacillus tropaeoli* sequence similarity 99% | EM II + M13 | nearly complete 16s rRNA gene sequencing (1518 bp) |
| 22 | B. lapidarius | Gentbrugge | Closest to *Lactobacillus kunkeei* sequence similarity 100% | EM I + M13 | nearly complete 16s rRNA gene sequencing (1539 bp) |
| 24 | *B. terrestris**lucorum* | Gentbrugge | Closest to *Lactobacillus kimchicus* sequence similarity 99% | EM I + M13 | nearly complete 16s rRNA gene sequencing (1546 bp) |
| 25 | *B. lapidarius* | Gentbrugge | Closest to *Leuconostoc citreum* sequence similarity 99% | EM I + M13 | nearly complete 16s rRNA gene sequencing (1516 bp) |
| 29 | B. terrestris\\lucorum | Bourgoyen | Closest to *Leuconostoc citreum* sequence similarity 99% | EM I + M13 | nearly complete 16s rRNA gene sequencing (1518 bp) |
| 29 | B. terrestris\\lucorum | Bourgoyen | Closest to *Lactococcus lactis* sequence similarity 100% | EM II + M13 | nearly complete 16S rRNA gene sequencing (1513 bp) |
| 66 | *B. pascuorum* | Bourgoyen | Closest to '*Commensalibacter intestini*' sequence similarity 99% | M13/M404 | MALDI–TOF MS\\ nearly complete 16S rRNA gene sequencing |

**Supplementary Table 4.2:** Cellular fatty acid contents (%) of *Bombella intestini* gen. nov., sp. nov. (data in bold) and type strains of all the type species of the family *Acetobacteraceae*.

1 = *Bombella intestini* LMG 28161$^{T}$, 2 = *Saccharibacter floricola* LMG 23170$^{T}$, 3 = *Acetobacter aceti* LMG 1504$^{T}$, 4 = *Acidomonas methanolica* LMG 1668$^{T}$, 5 = *Ameyamaea chiangmaiensis* LMG 27010$^{T}$, 6 = *Asaia bogorensis* LMG 21650$^{T}$, 7 = '*Commensalibacter intestini*'LMG 27436, 8 = *Endobacter medicaginis* LMG 26838$^{T}$, 9 = *Gluconacetobacter liquefaciens* LMG 1381$^{T}$, 10 = *Gluconobacter oxydans* LMG 1408$^{T}$, 11 = *Granulibacter bethesdensis* R–35628$^{T}$, 12 = *Komagataeibacter xylinus* LMG 1515$^{T}$, 13 = *Kozakia baliensis* LMG 21812$^{T}$, 14 = *Neoasaia chiangmaiensis* LMG 24037$^{T}$, 15 = *Neokomagataea thailandica* LMG 27021$^{T}$, 16 = *Swaminathania salitolerans* LMG 21291$^{T}$, 17 = *Tanticharoenia sakaeratensis* LMG 27022$^{T}$. –, not detectable or trace amount (<1%). All data were generated in the frame of this study. Cultivation conditions prior to fatty acid extraction were identical for all strains, except for the duration of the cultivation that varied from 48 hours to 72 hours depending on the strain, in order to obtain sufficient growth.

| | $C_{18:1}\omega7c$ | $C_{19:0}$ cyclo $\omega8c$ | $C_{16:0}$ | $C_{14:0}$ | $C_{14:0}$ 2–OH | $C_{16:0}$ 3–OH | $C_{16:0}$ 2–OH | $C_{18:1}$ 2–OH | $C_{18:0}$ | $C_{18:0}$ 3–OH | $C_{17:1}\omega6c$ | $C_{10:0}$ 3–OH | $C_{17:0}$ | $C_{19:0}$ 10–methyl |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 43.1 | 16.7 | 12.7 | 9.3 | 8.5 | 2.8 | 1.1 | – | – | – | – | – | – | – |
| 2 | 29.8 | 28.4 | 24.4 | 2.8 | – | 2.9 | 6.4 | – | 1.1 | – | – | – | – | – |
| 3 | 28.8 | – | 10.9 | 4.2 | 14.3 | 4.1 | 15.9 | – | 3.3 | 3.2 | – | – | – | – |
| 4 | 27.1 | – | 4.7 | – | 4.6 | 6.3 | 16.9 | 16.5 | – | 2.7 | – | 8.6 | – | – |
| 5 | 53.8 | 3.8 | 10.5 | – | 2.2 | 1.9 | 6.8 | 1.7 | 2.3 | – | 4 | – | 6.5 | – |
| 6 | 33.7 | – | 7.3 | – | 20.5 | 7.4 | 15.5 | 3.2 | – | 1.4 | – | – | – | – |
| 7 | 40.5 | – | 7.4 | 2.2 | – | 7.8 | 9.2 | 25.5 | 3.7 | – | – | – | – | – |
| 8 | 27.4 | – | 4.6 | – | – | 9.1 | 7 | 36.2 | 1.4 | 3.5 | 1.8 | – | – | – |
| 9 | 35.4 | 1.2 | 6.7 | 7.8 | 10.9 | 8.8 | 19.1 | 1.6 | – | 1.8 | – | – | – | – |
| 10 | 51 | – | 9.6 | – | – | 6.7 | 20.8 | – | 2.1 | 4.3 | – | – | – | – |
| 11 | 30.6 | – | 12 | 2.7 | 8.5 | 6.9 | 2 | 23.8 | – | 1.7 | 2.5 | – | – | – |
| 12 | 63.5 | – | 28 | – | – | – | 8.5 | – | – | – | – | – | – | – |
| 13 | 43.3 | 2 | 13.5 | 1.4 | 13.9 | 4.9 | 14.2 | – | – | 1.7 | – | – | – | – |
| 14 | 32.5 | 2 | 10.1 | 3.2 | 12 | 7.8 | 19.5 | 3.4 | – | 2.7 | – | – | – | – |
| 15 | 49.2 | – | 10.4 | – | – | 4.1 | 5.7 | 17.2 | 4.2 | 3.5 | – | – | – | 1.3 |
| 16 | 27.4 | 2 | 8.2 | 1.1 | 24.2 | 6.6 | 16 | 1.1 | 1 | 1.8 | – | – | – | – |
| 17 | 39.4 | – | 7.5 | 2.3 | 14.4 | 7.3 | 16.3 | 5.1 | 1 | 1.1 | – | – | – | – |

## 4.2 Novel lactic acid bacteria isolated from the bumblebee gut: *Convivina intestini* gen. nov., sp. nov., *Lactobacillus bombicola* sp. nov., and *Weissella bombi* sp. nov.

**Redrafted from:** Jessy Praet, Ivan Meeus, Margo Cnockaert, Kurt Houf, Guy Smagghe, Peter Vandamme (2015). Novel lactic acid bacteria isolated from the bumblebee gut: *Convivina intestini* gen. nov., sp. nov., *Lactobacillus bombicola* sp. nov., and *Weissella bombi* sp. nov. Antonie Van Leeuwenhoek, 107, 1337–1349.

**Author contributions:** JP and PV conceived the study and wrote the manuscript. JP and MC performed the experiments. KH, IM and GS proofread the manuscript.

### 4.2.1 Abstract

Twelve isolates of lactic acid bacteria (LAB) were obtained in the course of a bumblebee gut microbiota study and grouped into four matrix-assisted laser desorption/ionisation time–of–flight mass spectrometry clusters. Comparative 16S rRNA gene sequence analysis revealed that cluster 1 isolates, represented by strain LMG 28288$^{T}$, are most closely related to *Lactobacillus apis* (97.0% sequence similarity to that of *L. apis* LMG 26964$^{T}$). Cluster 2 isolates represented by strain LMG 28290$^{T}$ are most closely related to *Weissella hellenica* (99.6% sequence similarity to that of *W. hellenica* LMG15125$^{T}$). The single cluster 3 and 4 isolates had identical 16S rRNA gene sequences which were 94.8% similar to that of *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893$^{T}$, their nearest phylogenetic neighbour. A polyphasic taxonomic study additionally including comparative *pheS* sequence analysis, DNA–DNA hybridization experiments, DNA G+C content analysis, (GTG)$_5$–PCR fingerprinting and a biochemical characterization, demonstrated that cluster 1 isolates represent a novel *Lactobacillus* species for which we propose the name *Lactobacillus bombicola* sp. nov. with LMG 28288$^{T}$ (=DSM 28793$^{T}$) as the type strain; and that cluster 2 isolates represent a novel *Weissella* species for which we propose the name *Weissella bombi* sp. nov. with LMG 28290$^{T}$ (= DSM 28794$^{T}$) as the type strain. Cluster 3 and 4 isolates, in contrast, represented a very distinct, novel taxon that could be distinguished from members of the genera *Leuconostoc* and *Fructobacillus*, its nearest phylogenetic neighbours, by its cellular morphology, non-fructophilic metabolism and DNA G+C content. We therefore classify both isolates into a novel species representing a novel LAB genus for which the name *Convivina intestini* gen. nov., sp. nov. is proposed with LMG 28291$^{T}$ (= DSM 28795$^{T}$) as the type strain.

### 4.2.2 Introduction

Bumblebees are essential pollinators of wild plants and commercial crops, especially in the north temperate zone of the world. Although most commercial crops do not depend on insect pollination, it greatly improves the yield and the quality of many crops, such as tomatoes and sweet pepper. Therefore, insect pollination of commercial crops to which bumblebees contribute considerably, is of major value to agriculture [Gallai et al., 2009; Garibaldi et al., 2013]. The maintenance of bumblebee biodiversity is also important for ecosystem integrity of wild plants and, consequently, there is considerable anxiety about worldwide declines of bumblebee populations [Cameron et al., 2011; Goulson et al., 2006; Grixti et al., 2009; Rasmont et al., 2005; Williams et al., 2009; Williams, 1982]. These declines are presumably caused by a combination of factors with pesticide use, changes in agricultural practices and pathogen emergence commonly regarded to be the most important ones [Goulson et al., 2008b; Meeus et al., 2011]. Over the past 10 years, intestinal dysbiosis has gained attention to explain emergent diseases. A study of Cox-Foster et al. [2007] showed that the abundance of lactic acid bacteria (LAB) in diseased honeybees was significantly lower than in healthy bees. In addition, several studies indicated that LAB play a major role in the health of bumblebees and honeybees. They may prevent pathogens such as *Paenibacillus larvae*, *Melissococcus plutonius*, *Nosema bombi* and *Crithidia bombi* from causing diseases by either competing for the gut niche [Anderson et al., 2011] or by direct inhibition through the production of antimicrobial compounds such as hydrogen peroxide, organic acids and antimicrobial peptides and acidification of the gut environment [Audisio et al., 2011]. Although the bumblebee gut microbiota is only partially characterized, LAB and bifidobacteria belong to a core set of bumblebee gut bacteria [Olofsson et al., 2014; Killer et al., 2014; Koch and Schmid-Hempel, 2011b; Martinson et al., 2011; Mohr and Tebbe, 2006; Killer et al., 2009]. Metagenomic studies have demonstrated the presence of several phylotypes of LAB in the guts of honeybees and bumblebees [Koch and Schmid-Hempel, 2011b; Mohr and Tebbe, 2006; Olofsson and Vásquez, 2008] and several cultured representatives have been isolated, including members of the genera *Lactobacillus*, *Fructobacillus* and *Enterococcus* [Audisio et al., 2011; Endo and Salminen, 2013; Killer et al., 2013, 2014]. In the course of a bumblebee gut microbiota study we isolated several LAB which could not be allocated to formally named species.

### 4.2.3 Materials and methods

Sampling of bumblebees and preparation of cell suspensions

Bumblebees of *Bombus pascuorum*, *Bombus lapidarius*, *Bombus hypnorum* and *Bombus terrestris* were caught in the field in the region of Ghent, Belgium, and identified by their colour pattern. In addition, the cytochrome oxidase I (COI) gene sequence was determined for bumblebees identified as *B. lapidarius* and *B. terrestris*, which can be confused with other *Bombus* species based on colour pattern. The COI gene was amplified as described by Carolan et al. [2012] after Chelex® 100 resin (Bio-Rad) DNA-extraction on two bumblebee legs which were ground with a micropestle [Walsh et al., 1991]. The bumblebees were

immobilized at –20°C for 10 minutes and surface sterilized with 2.5% Umonium$^{38®}$ Master (Laboratoire Huckert's International, Brussels, Belgium) before dissecting out their crop and gut. The crops and guts were homogenized in 125 µl saline solution (0.1% peptone, 0.1% Tween 80, 0.85% NaCl) with a sterile micro–pestle. Afterwards, 125 µl of a 10% DMSO solution was added to the cell suspensions which were stored at –80 °C until further use.

Isolation of bumblebee gut bacteria and dereplication

Cell suspensions were serially diluted to $10^{-4}$ in physiological saline (0.85% NaCl), plated on MRS agar (Oxoid), modified trypticase phytone yeast extract (MTPY) agar [Rada and Petr, 2000] and all culture (AC) agar (Sigma-Aldrich) and incubated aerobically (MRS), anaerobically (MRS and MTPY) and microaerobically (AC) at 37°C. After 5 days, colonies were picked up from the agar plates and third generation axenic isolates were dereplicated by MALDI–TOF MS followed by curve–based data analysis [Ghyselinck et al., 2011] using the BioNumerics 5.1 software (Applied Maths, Sint–Martens-Latem, Belgium). Representative isolates of each MALDI–TOF MS cluster were selected for further identification.

Phylogenetic analysis

The 16S rRNA gene sequences and *pheS* sequences were determined as previously described [De Bruyne et al., 2007; Snauwaert et al., 2013]. EzTaxon–e [Kim et al., 2012] was used for the analysis of the 16S rRNA gene sequences and NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST) for the analysis of the *pheS* sequences. The MEGA5 software package was used to align the sequences obtained with the corresponding sequences of their phylogenetic neighbour species by MUSCLE and to obtain phylogenetic trees by using the maximum–likelihood method and the general time–reversible model with invariant sites [Tamura et al., 2011]. The robustness of the topology of the trees was estimated by bootstrap analysis with 100 replicates [Felsenstein, 1985]. MEGA5 was also used to calculate the sequence similarity values.

DNA G+C content and DNA–DNA hybridization

The DNA G+C content of the type strains and the DNA–DNA hybridization between strain LMG 28290$^{T}$ and the type strain of *Weissella hellenica* LMG 15125$^{T}$ were determined as previously described [Cleenwerck et al., 2008].

$(GTG)_5$–PCR fingerprinting

Genotypic fingerprints of all isolates were obtained via $(GTG)_5$–PCR as described by Gevers [2001] and analysed with the BioNumerics 5.1 software [De Vuyst et al., 2008]

Phenotypic analysis

The isolates and type strains of their closest relatives (*Lactobacillus apis* LMG 26964$^{T}$, *W. hellenica* LMG 15125$^{T}$ and *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893$^{T}$)

were routinely grown anaerobically on MRS agar at 37°C, except *W. hellenica* LMG 15125$^T$ which was routinally grown aerobically at 25°C. To test substrate utilization and enzyme activity, API 50CHL strips and APIZYM strips (bioMérieux) were inoculated with dense cell suspensions (McFarland 2 and 5, respectively) of the isolates. The API 50CHL strips were read after 5 days of incubation at 37°C. Gram–stain behaviour, endospore staining and verification of oxidase and catalase activity were performed using standard microbiological procedures [Macfaddinn, 1980]. Growth was determined in triplicate in MRS broth (Oxoid) at different temperatures (10, 15, 25, 37 and 45°C), pH values (pH 3, 5, 7 and 9) and NaCl concentrations (5, 6, 7 and 8%). Gas production from glucose was determined using inverted Durham tubes in MRS broth. Cell morphology and motility was checked with light microscopy and for LMG 28291$^T$ also with electron microscopy as described in Houf et al. [2009]. Verification of fructophilic growth characteristics of strains LMG 28291$^T$ and LMG 28625 was performed by growth on MRS agar and MRS agar supplemented with 2% fructose in aerobic and anaerobic atmospheres. Production of short chain fatty acids was determined after growth in MRS broth for 3 days as described by De Baere et al. [2013]. The D–/L–Lactic Acid (D–/L–Lactate) (Rapid) Assay Kit (Megazyme) was used to determine D– or L–lactic acid production.

### 4.2.4 Results

MALDI–TOF MS dereplication

LAB isolates obtained from the bumblebee gut samples assembled into several MALDI–TOF MS clusters; isolates of most of these clusters were identified as *Fructobacillus tropaeoli*, *Leuconostoc mesenteroides*, *Lactobacillus kunkeei*, *Enterococcus faecalis* and *Lactococcus lactis* based on the profiles of reference strains present in our in–house database (data not shown). However, the profiles of the isolates of four MALDI–TOF MS clusters (Figure 4.2) were different from those of the LAB in the in–house reference database (Supplementary Figure 4.7). Cluster 1 and 2 profiles originated from isolates from multiple bumblebee species each; in contrast, cluster 3 and 4 profiles originated from biological and technical replicates of a single isolate each. An overview of all isolates, their hosts and sampling details is provided in Table 4.3).

Phylogenetic analyses

Strains LMG 28288$^T$, LMG 28290$^T$, LMG 28291$^T$ and LMG 28625 were chosen as representatives of the four clusters. Analysis of their 16S rRNA gene sequences demonstrated that LMG 28288$^T$ and LMG 28290$^T$ are most closely related to *Lactobacillus apis* LMG 26964$^T$ (97.0% sequence similarity) and *W. hellenica* LMG 15125$^T$ (99.6% sequence similarity), respectively (Supplementary Figures 4.8 and 4.9). The 16S rRNA gene sequence of strain LMG 28288$^T$ was also 99.9% similar to that of a *Lactobacillus* phylotype (clone Q05008Plasm3 1486 bp) detected in a bumblebee gut metagenomics study [Koch and Schmid-Hempel, 2011b]. The 16S rRNA gene sequences of strains LMG28291$^T$ and LMG28625 were identical and showed

the highest sequence similarity (94.8%) to that of *L. mesenteroides* subsp. *mesenteroides* LMG6893$^T$; they were also 99% similar to that of an uncultured Firmicutes phylotype (murgBL2to) detected by Vasquez et al. [2012] in honeybees (Supplementary Figure 4.10). A 16S rRNA gene sequence based phylogenetic tree comprising strains LMG 28288$^T$, LMG 28290$^T$ and LMG 28291$^T$ and their nearest phylogenetic neighbour species is shown in (Figure 4.3). The *pheS* sequences of all six cluster 1 isolates were determined and revealed 98.9–100% sequence similarity to that of strain LMG 28288$^T$; the MEGA5 analysis confirmed *L. apis* as the nearest neighbour species and revealed 81.5% similarity to the *pheS* sequence of *L. apis* LMG 26964$^T$. The *pheS* sequences of all four cluster 2 isolates were identical; the MEGA5 analysis of the *pheS* sequence of strain LMG 28289$^T$ confirmed *W. hellenica* as the nearest neighbour species and revealed 89.% similarity to the *pheS* sequence of *W. hellenica* LMG 15125$^T$. Finally, the *pheS* sequences of strains LMG 28291$^T$ and LMG 28625 were identical and sequence analysis in MEGA5 showed the highest sequence similarity to the *pheS* sequence of *Fructobacillus ficulneus* LMG 21928$^T$ (81.5%). (Supplementary Figures 4.11, 4.12 and 4.13) present *pheS* sequence based phylogenetic trees of strains LMG 28288$^T$, LMG 28290$^T$ and LMG 28291$^T$ and some of their neighbouring taxa.

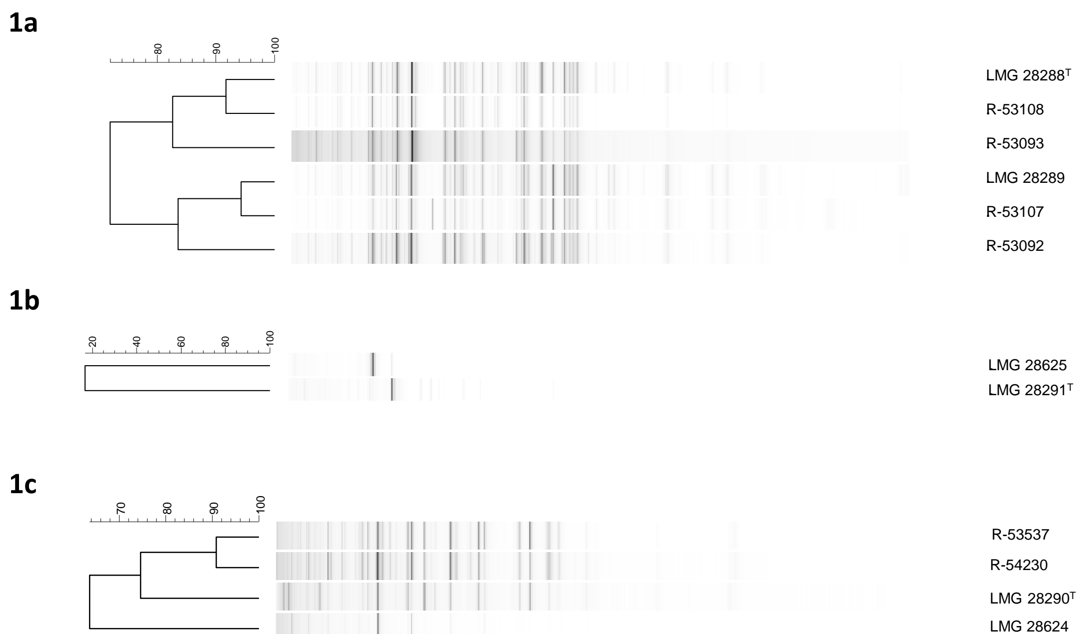



**Figure 4.2:** MALDI–TOF MS profiles of the *Lactobacillus bombicola* (a), *Convivina intestini* (b), and *Weissella bombi* isolates (c). Cluster analysis was performed by Pearson correlation and the hierarchical clustering method UPGMA.

**Table 4.3:** Isolates, their sources and MALDI–TOF MS cluster numbers.

| Isolates | Isolation source[a] | MALDI–TOF MS cluster |
|---|---|---|
| LMG 28288$^T$ | Bumblebee H70 *B. lapidarius* | 1 |
| | Bourgoyen Ghent (51.06840°NL/ 3.685100°EL) | |
| LMG 28289 | Bumblebee H53 *B. pascuorum* | |
| | Coupure Ghent (51.05130°NL/ 3.706°EL) | |
| R–53092 | Bumblebee H66 *B. pascuorum* | |
| | Bourgoyen Ghent (51.06840°NL/ 3.685100°EL) | |
| R–53093 | Bumblebee H113 *B. lapidarius* | |
| | Ledeganck Ghent (51.0368°NL/ 3.7221°EL) | |
| R–53107 | Bumblebee H87 *B. vestalis* | |
| | Den Blakken Wetteren (51.0086°NL/ 3.899100°EL) | |
| R–53108 | Bumblebee H18 *B. terrestris* | |
| | Ledeganck Ghent (51.0368°NL/ 3.7221°EL) | |
| LMG 28290$^T$ | Bumblebee H24 *B. terrestris* | 2 |
| | Gentbrugge (51.0462°NL/ 3.7608°EL) | |
| R–53537 | Bumblebee H1 *B. terrestris* | |
| | Ledeganck Ghent (51.0368°NL/ 3.7221 °EL) | |
| R–54230 | Bumblebee H15 *B. terrestris* | |
| | Ledeganck Ghent (51.0368°NL/ 3.7221 °EL) | |
| LMG 28624 | Bumblebee H69 *B. hypnorum* | |
| | Bourgoyen Ghent (51.06840°NL/ 3.685100 °EL) | |
| LMG 28291$^T$ | Bumblebee H79 *B. terrestris* | 3 |
| | Bourgoyen Ghent (51.06840°NL/ 3.685100°EL) | |
| LMG 28625 | Bumblebee H79 *B. terrestris* | 4 |
| | Bourgoyen Ghent (51.06840°NL/ 3.685100°EL) | |

[a] Isolation source (bumblebee individual, bumblebee species and sampling location).

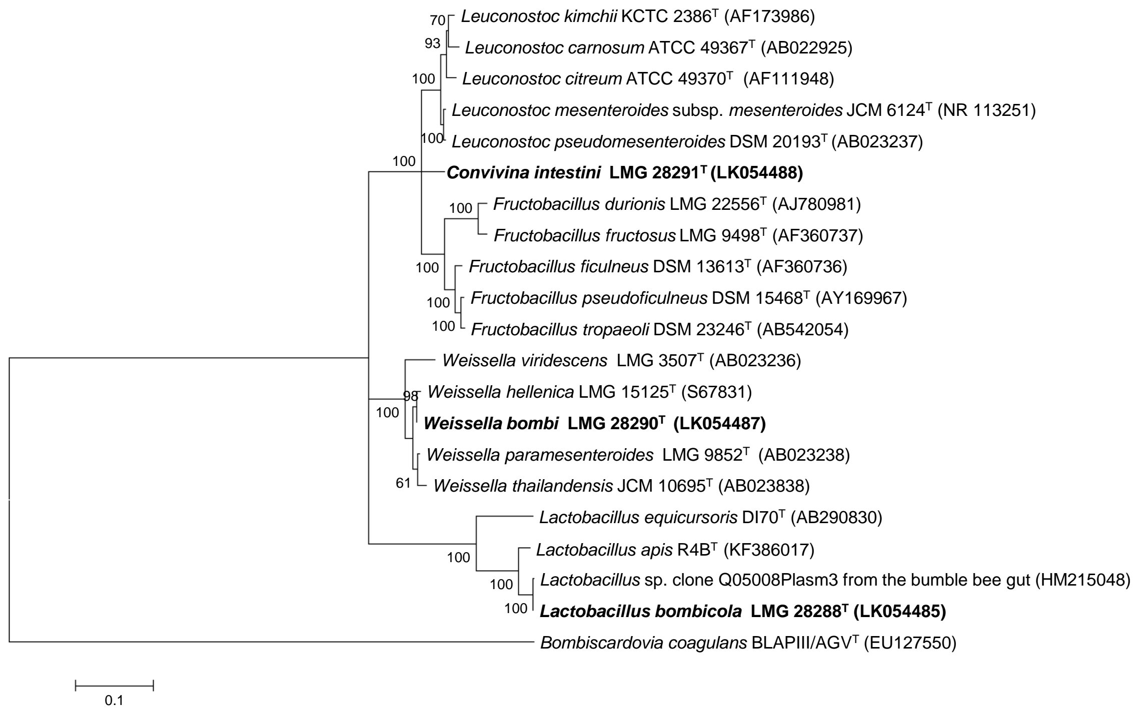



**Figure 4.3:** Restricted phylogenetic tree based on 16S rRNA gene sequences of *Lactobacillus bombicola* LMG 28288$^{T}$, *Weissella bombi* LMG 28290$^{T}$ and *Convivina intestini* LMG 28291$^{T}$ and established *Lactobacillus*, *Weissella*, *Fructobacillus* and *Leuconostoc* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analysed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage is 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.2022)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 57.0917 % sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. There were a total of 1575 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].

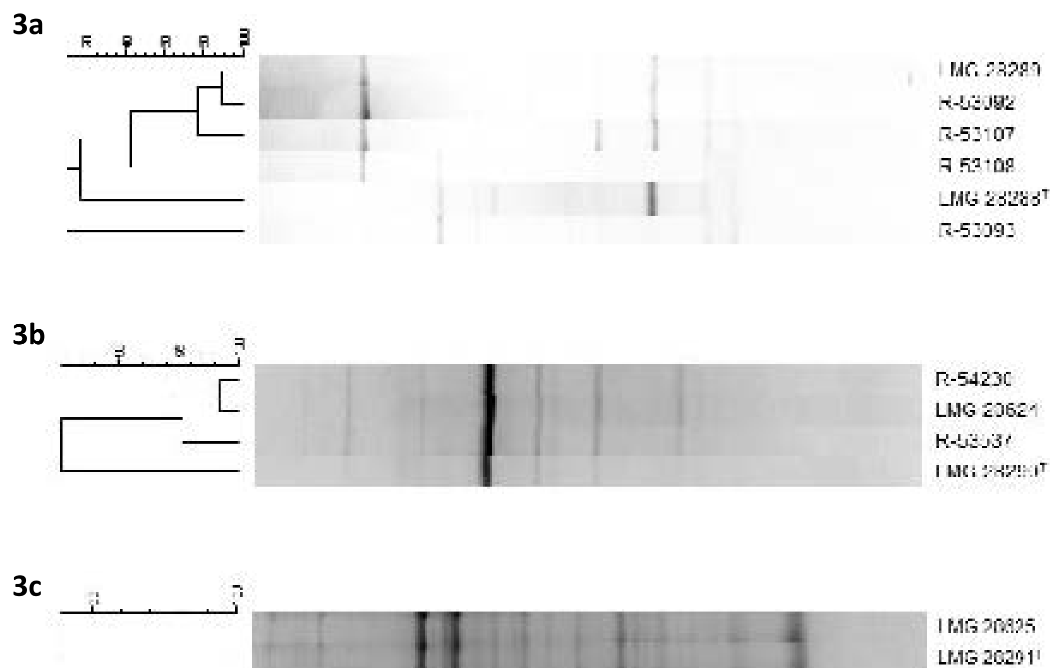



**Figure 4.4:** $(GTG)_5$–PCR profiles of different *Lactobacillus bombicola* (a), *Weissella bombi* (b) and *Convivina intestini* (c) isolates. Cluster analysis was performed by Pearson correlation and the hierarchical clustering method UPGMA

$(GTG)_5$–PCR–fingerprinting

The $(GTG)_5$–PCR profiles (Supplementary Figure 4.14) of two cluster 1 isolates (LMG 28289 and R–53092) were highly similar; all others were different (Figure 4.4 a). The $(GTG)_5$–PCR profile of one cluster 2 isolate (LMG 28290$^T$) differed slightly from those of the remaining three cluster 2 isolates (Figure 4.4 b). Finally, the $(GTG)_5$–PCR profiles of cluster 3 isolate LMG 28291$^T$ and cluster 4 isolate LMG 28625 were highly similar (Figure 4.4 c).

DNA–DNA hybridization and DNA G+C content analysis

DNA–DNA hybridizations were performed between strain LMG 28290$^T$ and *W. hellenica* LMG 15125$^T$. The DNA–DNA hybridization value was 37.2 ± 4.5% (the reciprocal values were 34.1 and 40.3%). The DNA G+C content of strains LMG 28288$^T$, LMG 28290$^T$ and LMG 28291$^T$ was 34.5, 37.2 and 31.9 mol%, respectively.

Phenotypic analyses

Phenotypic analyses were performed for all 12 isolates and the type strains of their closest neighbours (*L. apis* LMG 26964$^T$, *W. hellenica* LMG 15125$^T$ and *L. mesenteroides* subsp. *mesenteroides* LMG 6893$^T$) as determined through 16S rRNA gene sequencing, unless stated otherwise. All isolates were found to be facultative anaerobes and Gram–stain positive, non–motile, oxidase–and catalase–negative. They did not form endospores.
Cluster 1 isolates and *L. apis* LMG 26964$^T$ were found to produce acid from D–ribose, D–glucose, D–fructose, D–mannose, N–acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D–trehalose, gentiobiose and D–cellobiose. They did not produce acid from glycerol, erythritol, D–arabinose, D–and L–xylose, D–adonitol, methyl–$\beta$–D–xylopyranoside, D–galactose, L–sorbose, L–rhamnose, dulcitol, inositol, D–mannitol, D–sorbitol, methyl–$\alpha$–D–mannopyranoside, methyl–$\alpha$–D–glucopyranoside, D–maltose, D–lactose, D–melibiose, sucrose, inulin, D–melezitose, D–raffinose, starch, glycogen, xylitol, D–turanose, D–and L–fucose, D–arabitol, L–arabitol, potassium gluconate or potassium 2–gluconate. LMG 28288$^T$ and *L. apis* LMG 26964$^T$ exhibit activity of leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphtol–AS–BI–phosphohydrolase, $\beta$–glucosidase, N–acetyl–$\beta$–glucosaminidase. They do not exhibit activity of alkaline phosphatase, esterase lipase (C8), lipase (C14), trypsin, $\alpha$–chymotrypsin, $\alpha$–and $\beta$–galactosidase, $\beta$–glucuronidase, $\alpha$–glucosidase, $\alpha$–mannosidase and $\alpha$–fucosidase. Strain dependent and differential phenotypic characteristics between cluster 1 isolates and *L. apis* LMG 26964$^T$ are shown in Table 4.4.

Cluster 2 isolates and *W. hellenica* LMG 15125$^T$ were found to produce acid from L–arabinose, D–glucose, D–fructose, D–mannose, methyl–$\alpha$–D–glucopyranoside, N–acetylglucosamine, esculin ferric citrate, D–maltose, sucrose, D–trehalose and potassium gluconate but not from glycerol, erythritol, D–arabinose, D–and L–xylose, methyl–$\beta$ –D–xylopyranoside, L–sorbose, L–rhamnose, dulcitol, inositol, D–mannitol, D–sorbitol, methyl–$\alpha$–D–mannopyranoside, amygdalin, inulin, D–melezitose, starch, glycogen, xylitol, D–tagatose, D– and L–fucose or L–arabitol. LMG28290$^T$ and *W. hellenica* LMG 15125$^T$ exhibit activity of acid phosphatase, naphtol–AS–BI–phosphohydrolase, $\beta$–galactosidase and $\alpha$–glucosidase. They do not exhibit activity of esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, $\alpha$–chymotrypsin, $\alpha$–galactosidase, $\beta$–glucuronidase, $\beta$–glucosidase, N–acetyl–$\beta$–glucosaminidase, $\alpha$–mannosidase and $\alpha$–fucosidase. Strain dependent characteristics and differential phenotypic characteristics between cluster 2 isolates and *W. hellenica* LMG 15125$^T$ are shown in Table 4.4.

**Table 4.4:** Differential phenotypic characteristics of *Lactobacillus bombicola* LMG 28288$^T$ and *Lactobacillus apis* LMG 26964$^T$ and *Weissella bombi* LMG 28290$^T$ and *Weissella hellenica* LMG 15125$^T$.

| Phenotypic characteristic | LMG 28288$^T$ | LMG 26964$^T$ | LMG 28290$^T$ | LMG 15125$^T$ |
|---|---|---|---|---|
| Production of acid from: | | | | |
| L–Arabinose | + | − | + | + |
| D–Ribose | + | + | + (0/3)[a] | − |
| D–Adonitol | − | − | − (2/3)[a] | − |
| D–Galactose | − | − | + | w |
| Arbutin | + | + | + | − |
| Salicin | + | + | + (2/3)[a] | − |
| D–Cellobiose | + | +* | + | − |
| D–Lactose | − | − | + (2/3)[a] | − |
| D–Melibiose | − | − | + | − |
| D–Raffinose | − | − | + | − |
| Gentiobiose | + | + | + | − |
| D–Turanose | − | − | + | w |
| D–Lyxose | w | − | − | − |
| D–Tagatose | w | − | − | − |
| D–Arabitol | − | − | − | + |
| Potassium 2–ketogluconate | − | − | + (1/3)[a] | − |
| Potassium 5–ketagluconate | w | − | + | − |
| Alkaline phosphatase | − | − | − | + |
| Esterase (C4) | − | + | − | − |
| DNA G+C content (mol%) | 34.5 | 41.3 | 37.2 | 39.4 |
| Growth at pH 3 to 9 | pH 3–7 | pH 5–9 | pH 3–9 | pH 3–9 |
| Growth at 10°C to 45°C | 28°C–37°C | 28°C–37°C | 10°C–37°C | 10°C–28°C |

The API 50CHL and APIZYM test kits and the D–/L–lactic acid test (Megazyme) were performed to obtain the data. + positive, – negative, w weakly positive.

[a]Number of isolates with a reaction identical to that of the type strain.

*This result does not correspond with published data [Collins et al., 1993].

Biochemical characteristics of cluster 3 and 4 isolates are listed below. An electron microscopy picture of LMG 28291$^T$ cells is shown in Figure 4.5.

**Table 4.5:** Differential characteristics of the genera *Fructobacillus*, *Leuconostoc* and *Convivina*.

| Phenotypic characteristic | *Fructobacillus*[a] | *Leuconostoc*[b] | *Convivina* |
|---|---|---|---|
| Cell morphology | Rod shaped | Coccus | Rod shaped |
| Fructophilic metabolism | yes | no | no |
| % G+C | 42–45% | 37–44% | 31.9% |

[a]Data obtained from [Endo and Okada, 2008].

[b]Data obtained from [Vos et al., 2009].

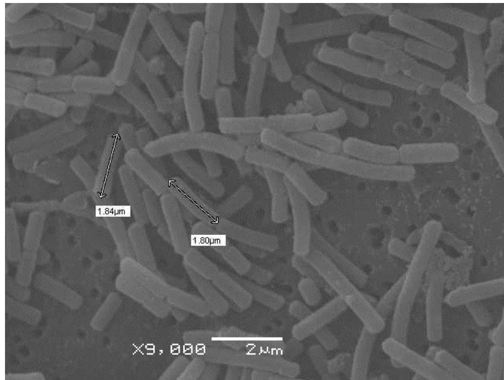



**Figure 4.5:** Electron microscopy picture of LMG 28291$^{T}$ cells.

### 4.2.5 Discussion

In the present study, LAB were isolated from the gut of bumblebees of *B. pascuorum*, *B. terrestris*, *B. vestalis*, *B. hypnorum* and *B. lapidarius*. Comparative MALDI–TOF MS analysis using an in–house database of LAB MALDI–TOF MS spectra allowed the assignment of most isolates to well–known LAB species such as *F. tropaeoli*, *L. mesenteroides*, *L. kunkeei*, *E. faecalis* and *L. lactis*, but revealed four clusters of spectra of isolates that could not be identified (Table 4.3).

The six cluster 1 isolates were obtained from six bumblebees representing four different species, i.e. *B. pascuorum*, *B. terrestris*, *B. vestalis* and *B. lapidarius* and were collected at different locations in the region of Ghent, Belgium (Table 4.3). The 16S rRNA gene and *pheS* sequences of strain LMG 28288$^{T}$ (Figure 4.3 and Supplementary Figure 4.11) revealed *L. apis* LMG 26964$^{T}$ as the nearest neighbour (97.0 and 81.5% sequence similarity, respectively). The value of the 16S rRNA gene sequence similarity is well below the species delineation cut–off values of 98% [Stackebrandt et al., 2014] or 98.65% [Kim et al., 2014] indicating that cluster 1 represented a novel *Lactobacillus* species. This novel species has most likely been observed earlier in a bumblebee gut metagenomic dataset of Koch and Schmid-Hempel [2011b] as revealed by a 16S rRNA gene sequence similarity of 99.9% between strain LMG 28288$^{T}$ and *Lactobacillus* clone Q05008Plasm3. It is noteworthy that several additional *Lactobacillus* phylotypes have been reported in the gut microbiota of honeybees and bumblebees, of which only some have been formally named (i.e. *L. kunkeei*, *Lactobacillus bombi*, *L. apis*, *Lactobacillus apinorum*, *Lactobacillus mellifer*, *Lactobacillus mellis*, *Lactobacillus melliventris*, *Lactobacillus kimbladii*, *Lactobacillus helsingborgensis* and *Lactobacillus kullabergensis*) [Endo et al., 2012; Killer et al., 2014, 2013; Olofsson et al., 2014]. The DNA G+C content of LMG 28288$^{T}$ (34.5 mol%) differs from that of *L. apis* (41.3 mol%). Although phenotypically coherent, the *pheS* sequences and $(GTG)_5$–PCR fingerprints revealed some genotypic differences among the six cluster 1 isolates indicating that multiple strains were isolated and studied. The present novel taxon can be distinguished from its nearest neighbour species, *L. apis*, both phenotypically (Table 4.4 and Supplementary Figure

4.7a) as well as genotypically (Figure 4.4). We therefore propose to classify the six cluster 1 isolates into the novel species *Lactobacillus bombicola* sp. nov., with strain LMG 28288$^{T}$ as the type strain and present its description below.

The four cluster 2 isolates originated from four bumblebees which represented two different species (*B. terrestris* and *B. hypnorum*) collected at three different locations (Table 4.3). Analysis of the 16S rRNA gene sequence of a representative isolate (LMG 28290$^{T}$) revealed its closest relatedness to *W. hellenica* LMG 15125$^{T}$ with 99.6% gene sequence similarity (Figure 4.3). DNA–DNA hybridization between LMG 28290$^{T}$ and *W. hellenica* LMG 15125$^{T}$ revealed a value of 37.2 ± 4.5% which indicated that LMG 28290$^{T}$ belongs to a novel species. The *pheS* gene sequences of all four isolates are identical and showed 89.2% similarity to that of *W. hellenica* LMG 15125$^{T}$. However, the $(GTG)_5$–PCR profile of LMG 28290$^{T}$ differed from those of the other three isolates. The DNA G+C content of LMG 28290$^{T}$ is 37.2 mol%, which differs from that of *W. hellenica* LMG 15125$^{T}$ (39.4 mol%). We are not aware of 16S rRNA gene sequences detected in metagenomic or other data sets of honeybees or bumblebees which are highly similar to the 16S rRNA gene sequences of the *Weissella* isolates of the present study. The cluster 2 isolates can be distinguished phenotypically as well as genotypically from its nearest neighbour species, *W. hellenica.* Therefore, we propose to classify them into the novel species *Weissella bombi* sp. nov., with strain LMG 28290$^{T}$ as the type strain.

Finally, the cluster 3 and 4 strains proved to represent a single taxon as revealed by their identical 16S rRNA gene and *pheS* sequences. This 16S rRNA gene sequence is 99% similar to that of an uncultured Firmicutes phylotype (murgBL2to) detected by Vasquez et al. [2012] in honeybees. The nearly complete 16S rRNA gene sequences demonstrated that this taxon occupies a phylogenetic position intermediate to that of the genera *Leuconostoc* and *Fructobacillus* (Figure 4.3). While EzTaxon–e analysis of the 16S rRNA gene sequence revealed a *Leuconostoc* as nearest neighbour species (i.e. *L. mesenteroides* subsp. *mesenteroides* LMG 6893$^{T}$; Figure 4.3), MEGA5 analysis of the *pheS* sequence revealed a *Fructobacillus* as the nearest neighbour species (i.e. *F. ficulneus* LMG 21928$^{T}$, Figure 4.13). However, the clearly rod–shaped cellular morphology (Figure 4.5) demonstrated that this taxon does not conform to the characteristics of *Leuconostoc* species, while the absence of a fructophilic metabolism differentiated it from the members of the genus *Fructobacillus* (Table 4.5). Indeed, *Fructobacillus* species prefer fructose as a carbon source and can only use glucose in an anaerobic atmosphere if an electron acceptor (such as fructose) is present [Endo et al., 2012; Endo and Salminen, 2013]. Strains LMG 28291$^{T}$ and LMG 28625, in contrast, grow rapidly with glucose as a carbon source in both aerobic as well as anaerobic conditions, and do not require fructose or an alternative electron acceptor for cultivation in an anaerobic atmosphere. Furthermore, the DNA G+C content of LMG 28291$^{T}$ is 31.9 mol% which is considerably different from the DNA G+C content range within the genera *Leuconostoc* (37–44 mol%; [Vos et al., 2009]) and *Fructobacillus* (42–45 mol %; [Endo and Okada, 2008]). Given the considerable phylogenetic divergence between this novel taxon and the genera

*Leuconostoc* and *Fructobacillus*, and the differential genotypic and phenotypic characteristics, we feel it is most appropriate to classify the taxon represented by strains LMG 28291$^{T}$ and LMG 28625 as a novel species of a novel LAB genus, for which we propose the name *Convivina intestini* gen. nov., sp. nov. with LMG 28291$^{T}$ as the type strain of the type species.

### 4.2.6 Description of *Lactobacillus bombicola* sp. nov.

*Lactobacillus bombicola* [bom.bi'co.la. L. n. *bombus* a boom, a deep hollow noise, buzzing, also the zoological genus name of the bumblebee; L. suf. –cola (derived from *incola*, inhabitant) dwelling, occurring in; N.L. n. *bombicola* occurring in *Bombus*].

Grows on MRS agar at 37°C and is facultative anaerobic, Gram–stain positive, non–motile and oxidase– and catalase–negative. Does not form endospores. The cells are rod shaped (0.5–1 µm wide and 3 µm long) and the colonies are brown, shiny, undulate and 2 mm after 2 days of incubation. Grows at 25 and 37°C but not at 10, 15 and 45°C. Grows at pH3–7 and does not produce gas from glucose. Only D–lactic acid is produced from glucose. No growth is observed at the tested NaCl concentrations (5–8 % NaCl). The type strain produces acid from D–cellobiose, L–arabinose, D–ribose, D–glucose, D–fructose, D–mannose, amygdalin, arbutin, D–trehalose, N–acetylglucosamine, esculin ferric citrate, salicin, D–lyxose (although weakly), D–tagatose (although weakly), potassium 5–ketogluconate (although weakly) and gentiobiose. The type strain does not produce acid from glycerol, erythritol, D–arabinose, D–xylose, L–xylose, D–adonitol, methyl–$\beta$–D–xylopyranoside, D–galactose, L–sorbose, L–rhamnose, dulcitol, inositol, D–mannitol, D–raffinose, inulin, D–melezitose, D–sorbitol, methyl–$\alpha$–D–mannopyranoside, methyl–$\alpha$–D–glucopyranoside, D–maltose, D–lactose, D–melibiose, sucrose, starch, glycogen, xylitol, D–turanose, D–fucose, L–fucose, D–arabitol, L–arabitol, potassium gluconate and potassium 2–ketogluconate. The type strain exhibits activity of leucine arylamidase, valine arylamidase, cysteine arylamidase, acid phosphatase, naphtol–AS–BI–phosphohydrolase, $\beta$–glucosidase, N–acetyl–$\beta$–glucosaminidase. The type strain does not exhibit activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), trypsin, $\alpha$–chymotrypsin, $\alpha$– and $\beta$–galactosidase, $\beta$–glucuronidase, $\alpha$–glucosidase, $\alpha$–mannosidase and $\alpha$–fucosidase. The DNA G+C content of the type strain is 34.5 mol%. The type strain, LMG 28288$^{T}$ (=DSM 28793$^{T}$), was isolated from the gut of a *Bombus lapidarius* bumblebee in 2013 in the region of Ghent, Belgium. The accession numbers for the 16S rRNA gene and *pheS* sequences of *L. bombicola* LMG 28288$^{T}$ are LK054485 and LM999917, respectively.

### 4.2.7 Description of *Weissella bombi* sp. nov.

*Weissella bombi* (bom'bi. L. n. *bombus* a boom, a deep hollow noise, buzzing, also the zoological genus name of the bumblebee; N.L. gen. n. *bombi* of *Bombus*, of a bumblebee).

Strains are facultative anaerobes and are Gram–stain positive, non–motile and oxidase– and catalase–negative. Do not form endospores. The cells are elongated cocci (0.5–1 µm

wide and 2 µm long) which occur in pairs or chains and the colonies are white, undulate, shiny and 1–2 mm after 2 days of incubation. The cells tend to precipitate in MRS broth. Growth is observed at pH 3–9 and 10–37°C but not at 45°C. Grows in the presence of the tested NaCl concentrations (5–8 % NaCl) and produces gas, D–lactic acid and acetic acid as end products from glucose fermentation. The type strain produces acid from L–arabinose, D–ribose, D–galactose, D–glucose, D–fructose, D–mannose, arbutin, D–trehalose, methyl–$\alpha$–D–glucopyranoside, N–acetylglucosamine, esculin ferric citrate, D–maltose, D–melibiose, D–raffinose, gentiobiose, D–lactose, sucrose, salicin, D–turanose, D–cellobiose, potassium gluconate, potassium 2–ketogluconate and potassium 5–ketogluconate. Acid is not produced from glycerol, erythritol, D–arabinose, D– and L–xylose, D–adonitol, methyl–$\beta$–D–xylopyranoside, L–sorbose, L–rhamnose, dulcitol, inositol, D–mannitol, amygdalin, inulin, D–melezitose, D–sorbitol, methyl–$\alpha$–D–mannopyranoside, starch, glycogen, xylitol, D–tagatose, D–fucose, L–fucose, D–arabitol and L–arabitol. The type strain exhibits activity of acid phosphatase, naphtol–AS–BI–phosphohydrolase, $\beta$–galactosidase and $\alpha$–glucosidase. The type strain does not exhibit activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, $\alpha$–chymotrypsin, $\alpha$–galactosidase, $\beta$–glucuronidase, $\beta$–glucosidase, N–acetyl–$\beta$–glucosaminidase, $\alpha$–mannosidase and $\alpha$–fucosidase. The DNA G+C content of the type strain is 37.2 mol%. The type strain LMG 28290$^{T}$ (=DSM 28794$^{T}$), was isolated from the gut of a *B. terrestris* bumblebee in 2013 in the region of Ghent, Belgium. The accession numbers for the 16S rRNA gene and *pheS* sequences of *W. bombi* LMG 28290$^{T}$ are LK054487 and LM999920, respectively.

### 4.2.8 Description of *Convivina* gen. nov.

Convivina (Con.vi.vi'na. L. n. *conviva* guest, table companion; N.L.fem. n. *Convivina* a commensal bacterium)

Cells are rod shaped, catalase– and oxidase–negative, Gram–stain positive, produce D–lactic acid and acetic acid as end products from glucose fermentation and do not form endospores. Non–fructophilic. *C. intestini* is the type species of the genus.

### 4.2.9 Description of *Convivina intestini* sp. nov.

*Convivina intestini* (in.tes.ti'ni. L. gen. n. *intestini*, of the gut)

The characteristics are as described for the genus with the following additional properties. The colonies are white, round, 1–2 mm, convex and shiny after 2 days. The cells are 0.5–1 µm wide and 2 µm long. Grows at pH 3–9 and at 10–45°C, although growth at 45°C is weak. Produces gas from glucose and grows weakly at the tested NaCl concentrations (5–8% NaCl) after 5 days of incubation. Acid is produced from D–ribose, D–glucose, D–fructose, D–mannitol, D–trehalose, esculin ferric citrate, sucrose and potassium gluconate. Acid is not produced from glycerol, erythritol, D–arabinose, L–arabinose, D–xylose, L–xylose, D–adonitol, methyl–$\beta$–D–xylopyranoside, D–galactose, D–mannose, L–sorbose, L–rhamnose, dulcitol, inositol, amygdalin, arbutin, D–raffinose, inulin, D–melezitose, D–sorbitol, methyl–$\alpha$–D–mannopyranoside, methyl–$\alpha$–D–glucopyranoside, N–acetylglucosamine, salicin, D–maltose, D–lactose, D–melibiose, starch, glycogen, xylitol, gentiobiose, D–raffinose, D–turanose, D–lyxose, D–tagatose, D–fucose, L–fucose, D–arabitol, L–arabitol, D–cellobiose, potassium 2–ketogluconate and potassium 5–ketogluconate. The type strain exhibits activity of alkaline phosphatase, leucine arylamidase, acid phosphatase and naphtol–AS–BI–phosphohydrolase. The type strain does not exhibit activity of esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, $\alpha$–chymotrypsin, $\alpha$– and $\beta$–galactosidase, $\beta$–glucuronidase, $\alpha$– and $\beta$–glucosidase, N–acetyl–$\beta$–glucosaminidase, $\alpha$–mannosidase and $\alpha$–fucosidase. The DNA G+C content of the type strain is 31.9 mol%.
The type strain LMG 28291$^{T}$ (=DSM 28795$^{T}$) was isolated from the gut of a *B. terrestris* bumblebee in 2013 in the region of Ghent, Belgium. The accession numbers for the 16S rRNA gene and *pheS* sequences of *C. intestini* LMG 28291$^{T}$ are LK054488 and LM999919, respectively.

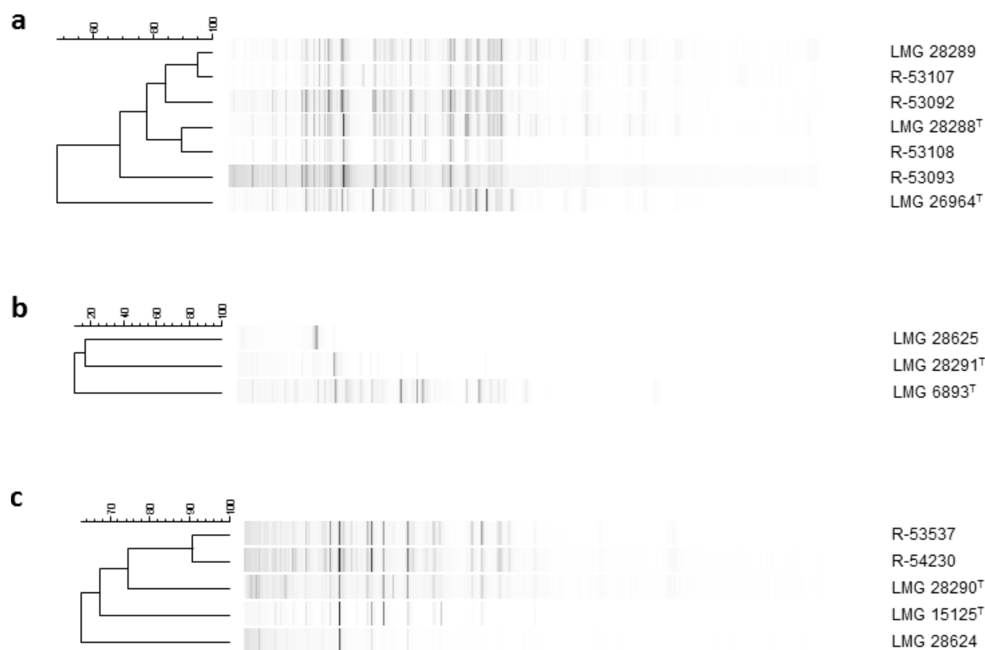
### 4.2.10 Acknowledgments

The authors acknowledge support by Ghent University-Special Research Fund (BOF).

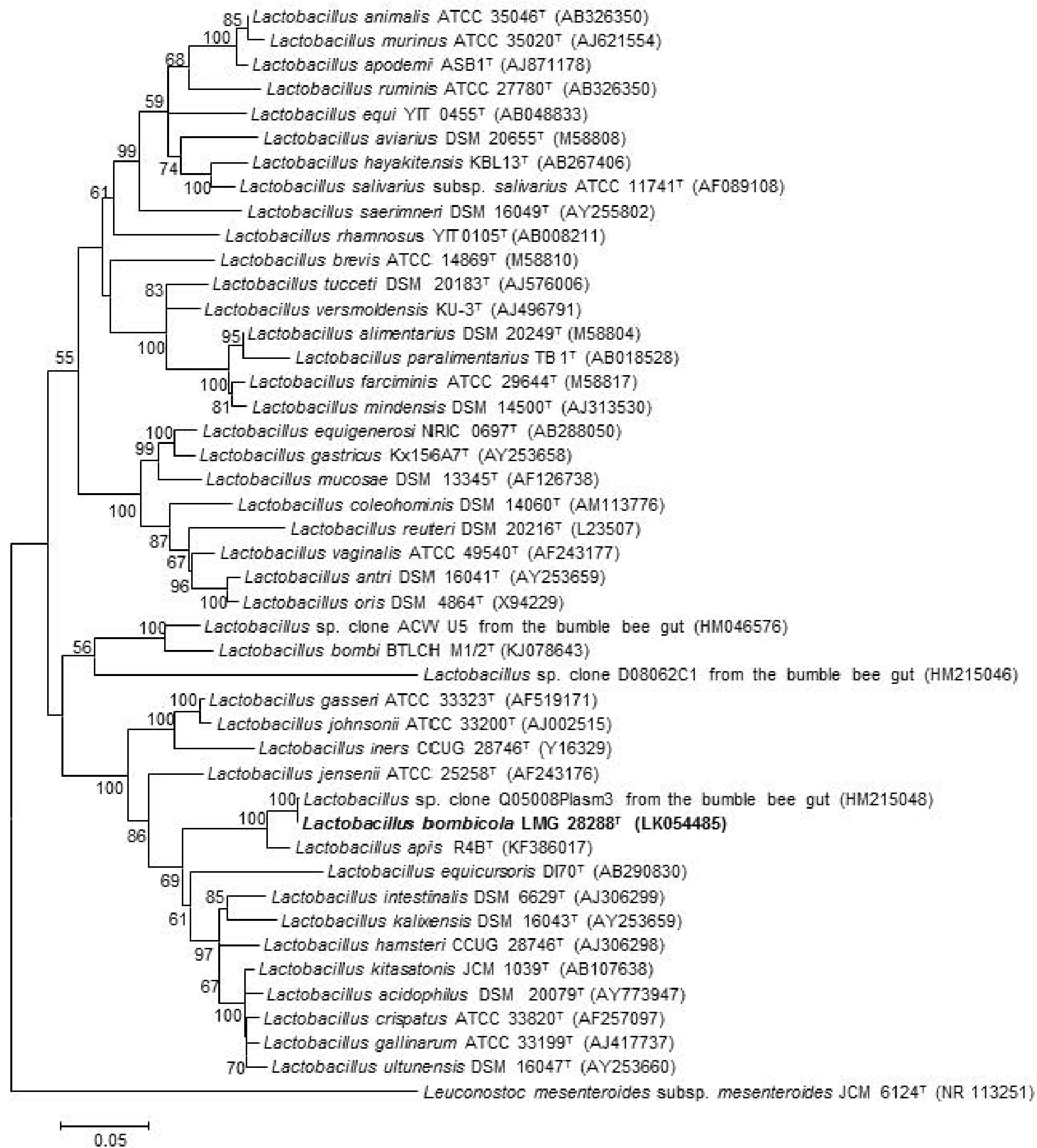
### 4.2.11 Compliance with Ethical Standards

The present research involved sampling of bumblebees for which no permission was required as bumblebees are not included in the ''Decree of Species (het Soortenbesluit (http://codex.vlaanderen.be/ Zoeken/Document.aspx?DID=1018227¶m=informatie])''of the Flemish government with inception on 01/09/2009. The authors do not have a conflict of interest.

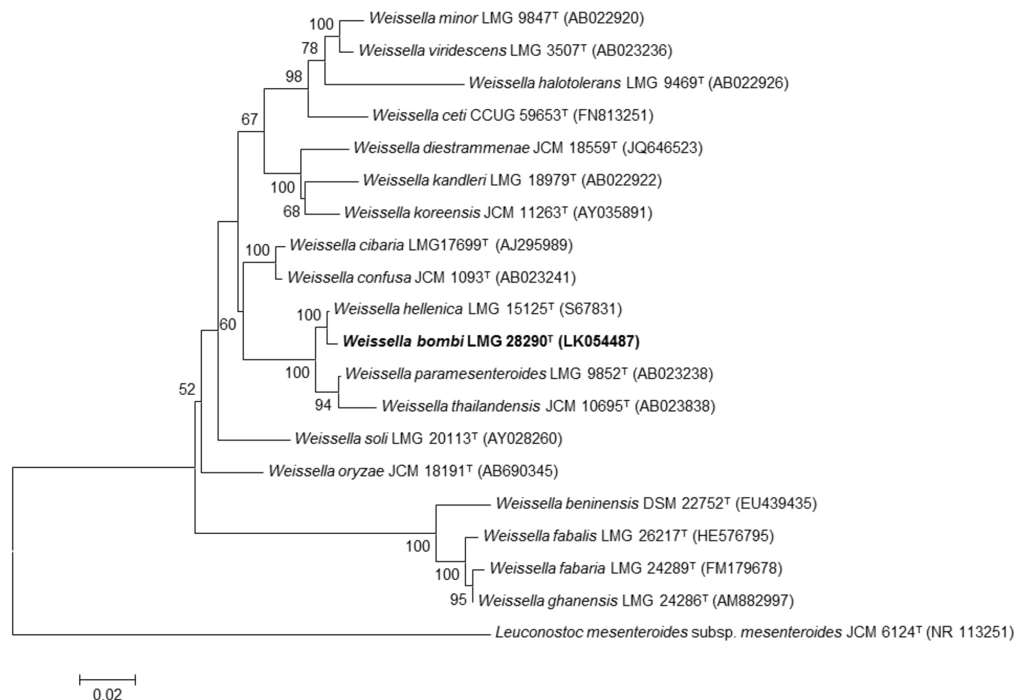
### 4.2.12 Supplementary material



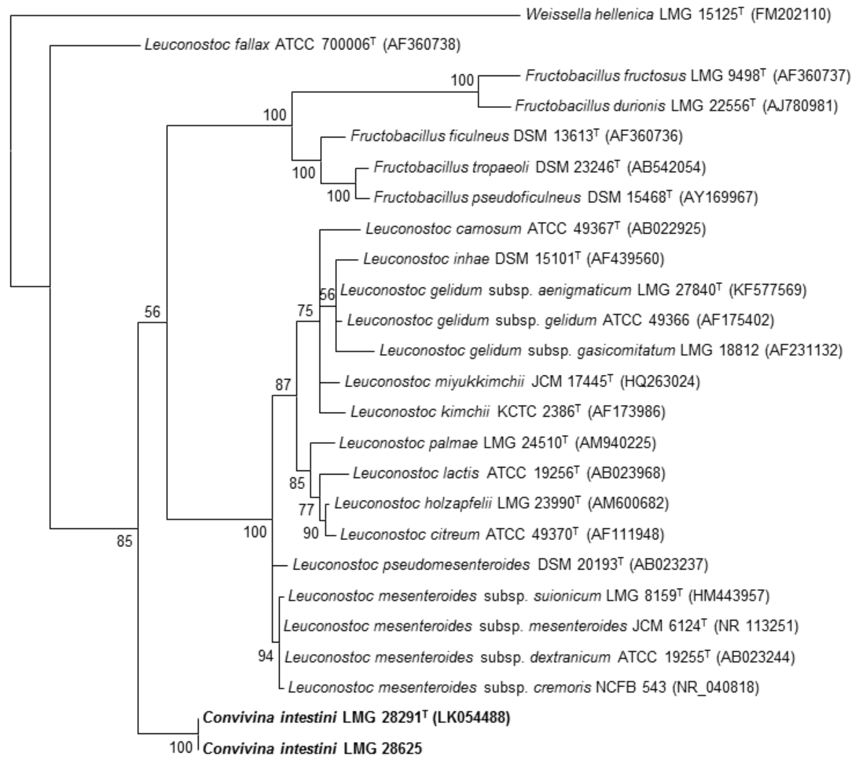

**Supplementary Figure 4.7:** MALDI–TOF MS profiles of the *Lactobacillus bombicola* (a), *Convivina intestini* (b), and *Weissella bombi* isolates (c) and their closest neighbours (*Lactobacillus apis* LMG 26964$^{T}$, *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893$^{T}$ and *Weissella hellenica* LMG 15125$^{T}$, respectively). Cluster analysis was performed by Pearson correlation and the hierarchical clustering method UPGMA.



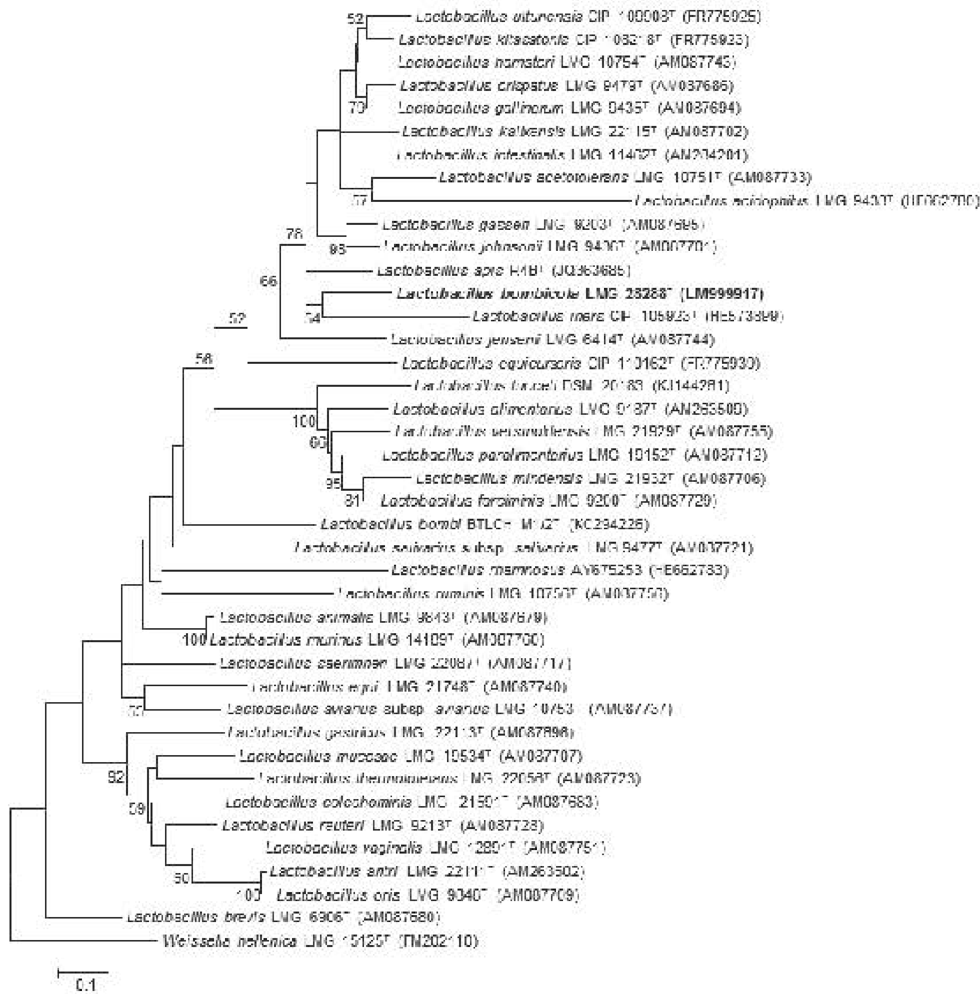

**Supplementary Figure 4.8:** Phylogenetic tree based on the 16S rRNA gene sequences of intestinal *Lactobacillus* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates [Felsenstein, 1985] is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches [Felsenstein, 1985] if the percentage was 50% or higher. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2353)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 50.1753% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences with *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124$^{T}$ as the outgroup. There were a total of 1629 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].



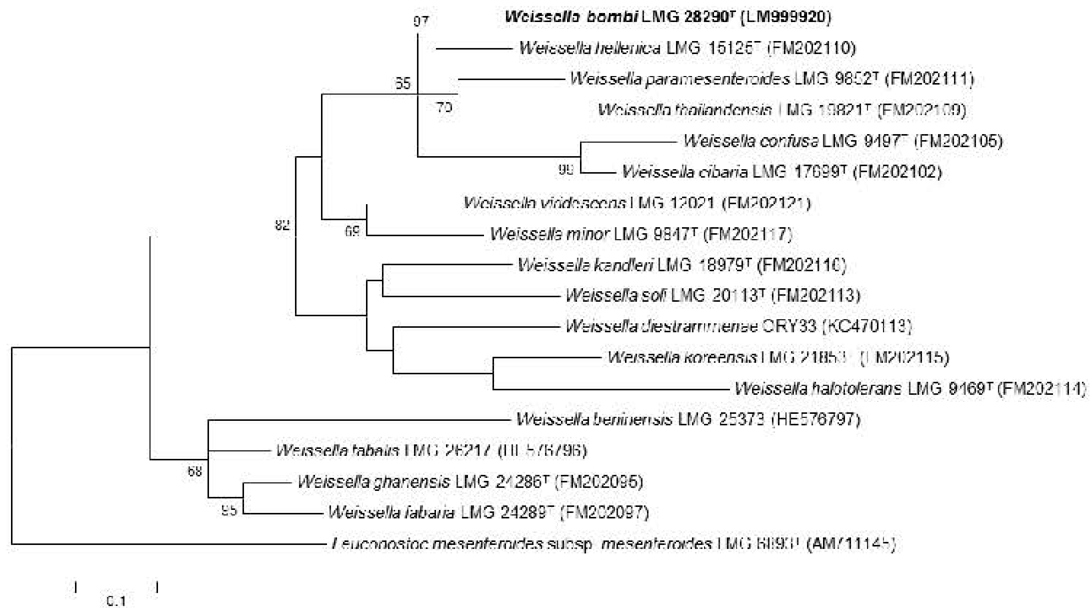

**Supplementary Figure 4.9:** Phylogenetic tree based on the 16S rRNA gene sequences of all *Weissella* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates [Felsenstein, 1985] is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage was 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1474)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 61.6388% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences with *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124$^{T}$ as the outgroup. There were a total of 1593 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].



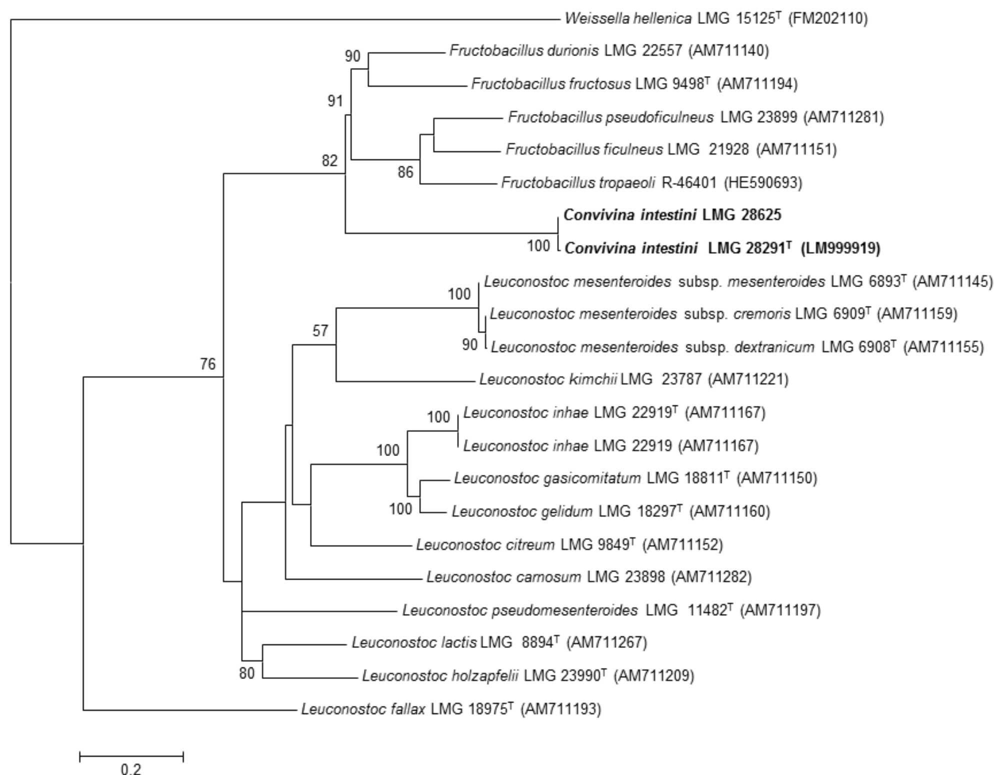

**Supplementary Figure 4.10:** Phylogenetic tree based on the 16S rRNA gene sequences of all *Leuconostoc* and *Fructobacillus* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates [Felsenstein, 1985] is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage was 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1379)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 66.0191% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 25 nucleotide sequences with *Weissella hellenica* LMG 15125$^{T}$ as the outgroup. There were a total of 1596 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].



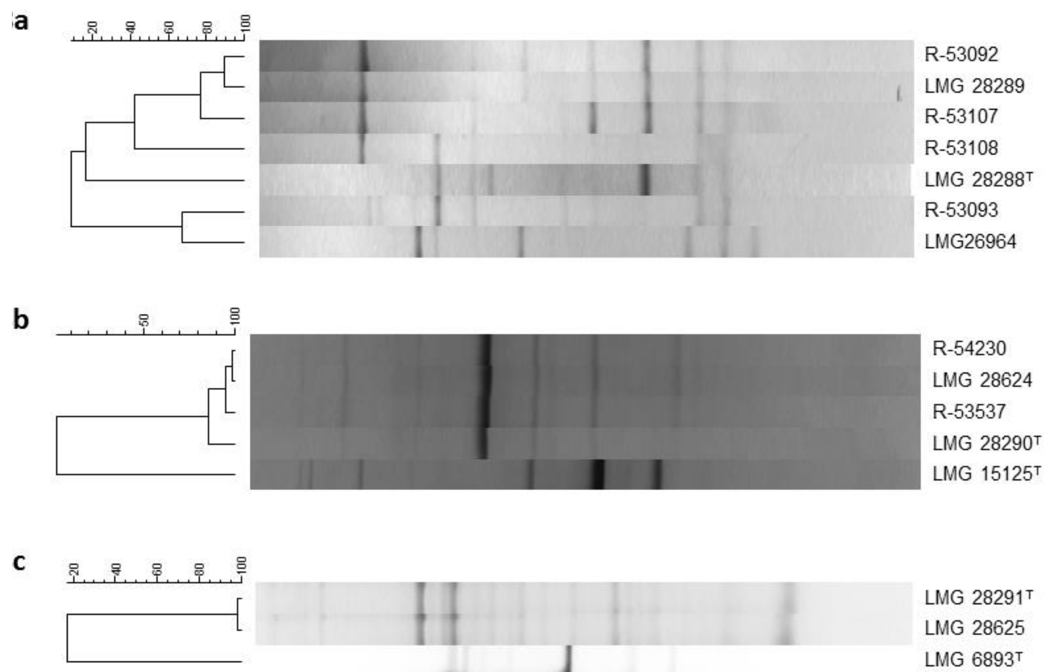

**Supplementary Figure 4.11:** Phylogenetic tree based on the *pheS* sequences of intestinal *Lactobacillus* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates [Felsenstein, 1985] is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage was 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7529)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 24.5718% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 41 nucleotide sequences with *Weissella hellenica* LMG 15125$^{T}$ as the outgroup. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 475 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].




**Supplementary Figure 4.12:** Phylogenetic tree based on the *pheS* sequences of all *Weissella* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates [Felsenstein, 1985] is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage was 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3810)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 38.8491% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences with *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893$^{T}$ as the outgroup. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 459 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].




**Supplementary Figure 4.13:** Phylogenetic tree based on the *pheS* sequences of all *Leuconostoc* and *Fructobacillus* species. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates [Felsenstein, 1985] is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage was 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.4716)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 32.3665% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 nucleotide sequences with *Weissella hellenica* LMG 15125$^{T}$ as the outgroup. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 471 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].




**Supplementary Figure 4.14:** $(GTG)_5$–PCR profiles of different *Lactobacillus bombicola* (a), *Weissella bombi* (b) and *Convivina intestini* (c) isolates and their closest neighbours (*Lactobacillus apis* LMG 26964$^T$, *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893$^T$ and *Weissella hellenica* LMG 15125$^T$, respectively). Cluster analysis was performed by Pearson correlation and the hierarchical clustering method UPGMA.

## 4.3 *Bifidobacterium commune* sp. nov. isolated from the bumblebee gut

**Redrafted from:** Jessy Praet, Ivan Meeus, Margo Cnockaert, Maarten Aerts, Guy Smagghe, Peter Vandamme (2015). *Bifidobacterium commune* sp. nov. isolated from the bumblebee gut. Antonie Van Leeuwenhoek, 107, 1307–1313.

**Author contributions:** JP and PV conceived the study and wrote the manuscript. JP, MC and MA performed the experiments. IM and GS proofread the manuscript.

### 4.3.1 Abstract

*Bifidobacteria* were isolated from the gut of *Bombus lapidarius*, *Bombus terrestris* and *Bombus hypnorum* bumblebees by direct isolation on modified trypticase phytone yeast extract agar. The MALDI–TOF MS profiles of four isolates (LMG 28292$^T$, R-53560, R-53124, LMG 28626) were found to be identical and did not cluster with the profiles of established *Bifidobacterium* species. Analysis of the 16S rRNA gene sequence of strain LMG 28292$^T$ revealed that LMG 28292$^T$ is most closely related to the *Bifidobacterium bohemicum* type strain (96.8%), which was also isolated from bumblebee gut specimens. The *hsp60* gene of strain LMG 28292$^T$ shows 85.8% sequence similarity to that of the *B. bohemicum* type strain. The $(GTG)_5$–PCR profiles and the *hsp60* sequences of all four isolates were indistinguishable; however, three different phenotypes were observed among the four isolates by means of the API 50CHL microtest system. Based on the phylogenetic, genotypic and phenotypic data, we propose to classify the four isolates within the novel species *Bifidobacterium commune* sp. nov., with LMG 28292$^T$ (= DSM 28792$^T$) as the type strain.

### 4.3.2 Introduction

Bumblebee populations are in worldwide decline in local range extent [Cameron et al., 2011; Goulson et al., 2006; Grixti et al., 2009; Rasmont et al., 2005; Williams, 1982]. These declines are presumably caused by a combination of factors, which differ between countries [Cameron et al., 2011; Williams et al., 2009] but the most important factors are generally considered the emergence of pathogens, intensified agricultural practices and pesticide use. As bumblebees are important pollinators of commercial crops and wild plants, their declines may have a strong impact on agriculture and ecosystem integrity. In Europe, the area of occupancy of several bumblebee species has been declining (e.g. *Bombus alpinus*, *Bombus caliginosus* and *Bombus fraternus*) (http://www.iucnredlist.org/search). To avoid or reverse these declines, conservation measures have to be developed. Recent studies have shown that the gut microbiota may contribute to honeybee and bumblebee health [Cariveau et al., 2014; Crotti et al., 2013; Endo and Salminen, 2013; Endo et al., 2012; Hamdi et al., 2011]. The gut microbiota of honeybees and bumblebees consists of a distinctive set of bacterial species including lactic acid bacteria (e.g. *Lactobacillus apis*, *Lactobacillus bombi*, *Lactobacillus kunkeei*, *Lactobacillus johnsonii*, *Fructobacillus fructosus* and *Enterococcus faecium*) and bifidobacteria (*Bifidobacterium actinocoloniiforme*, *Bifidobacterium bohemicum* and *Bifidobacterium bombi*) [Audisio et al., 2011; Endo and Salminen, 2013; Killer et al., 2009, 2013, 2011]. Several strains of these lactic acid bacteria inhibit the bee pathogens *Melissococcus plutonius* and *Paenibacillus larvae* [Audisio et al., 2011; Endo and Salminen, 2013] and promote the digestion of carbohydrates [Engel et al., 2012]. Hence, these bacteria are potential candidates as bumblebee probiotics. The present study reports on the characterization of a novel *Bifidobacterium* species which was isolated in the course of a bumblebee gut microbiota study.

### 4.3.3 Material and methods

Sampling of bumblebees and preparation of cell suspensions

*Bombus lapidarius*, *Bombus terrestris* and *Bombus hypnorum* bumblebees were caught in 2013 in the field in Ghent, Belgium, and identified by their colour patterns. In addition, the cytochrome oxidase I (COI) gene sequence was determined for bumblebees identified as *B. lapidarius* and *B. terrestris* which can be confused with other *Bombus* species based on colour patterns. The COI gene was amplified as described by Carolan et al. [2012] after Chelex® 100 resin (Bio–Rad) DNA–extraction on two bumblebee legs which were ground with a micropestle [Walsh et al., 1991]. The bumblebees were immobilized at –20°C for 10 min and surface sterilized with 2.5% Umonium[38]® Master (Laboratoire Huckert's International, Brussels, Belgium) before dissecting out their crop and gut. The crops and guts were homogenized in 125 µl saline solution (0.1% peptone, 0.1% tween 80, 0.85% NaCl) with a sterile micro–pestle. Afterwards, 125 µl of a 10% DMSO solution was added to the cell suspensions which were stored at –80°C until further use [Hoefman et al., 2012].

Isolation of bumblebee gut bacteria, cultivation and dereplication

Cell suspensions were serially diluted to $10^{-4}$ in physiological saline (0.85% NaCl), plated on modified trypticase phytone yeast extract (MTPY) agar [Rada and Petr, 2000] and incubated anaerobically at 37°C. After 5 days, colonies were picked from the agar plates and third generation axenic isolates were dereplicated by matrix–assisted laser desorption/ionisation–time–of–flight mass spectrometry (MALDI–TOF MS) followed by curve–based data analysis [Ghyselinck et al., 2011] using the BioNumerics 5.1 software (Applied Maths, Sint–Martens–Latem, Belgium). Representative isolates of each MALDI–TOF MS cluster were selected for further identification and were routinely incubated anaerobically on MTPY agar at 37°C unless indicated otherwise.

Phylogenetic analyses

The 16S rRNA gene sequence of strain LMG 28292$^T$ was determined as previously described [Snauwaert et al., 2013]. The forward primer MO–157 (5'–CTGGT GAAGGAGGTCGCCAA–3') and the reverse primer MO–158 (5'–CCATATCCTGCAGCATAGCCTT–3') were used for *hsp60* gene sequence analysis of the isolates LMG 28292$^T$, R-53560, R-53124 and LMG 28626 [Okamoto et al., 2008]. The *hsp60* gene amplification PCR mixture consisted of 2.5 µl 10x PCR–buffer (Applied Biosystems), 2.5 µl dNTPs (2 mM each, Applied Biosystems), 0.625 µl Taq polymerase (1 U/µl, Applied Biosystems), 0.25 µl of each primer (50 µM, Sigma–Aldrich), 2.5 µl of the alkaline lysate and milliQ to obtain a 25 µl PCR mixture. After an initial denaturation step of 2 minutes at 95°C, 30 cycles were performed of 30'' denaturation at 95°C, 30'' hybridization at 46°C and 30'' of elongation at 72°C. The PCR reaction was terminated by a 5' elongation step at 72°C. EzTaxon–e [Kim et al., 2012] was used for the analysis of the 16S rRNA gene sequence and NCBI BLAST (www.ncbi.nlm.nih.gov/BLAST) for the analysis of the *hsp60* gene sequences. The MEGA5 software package was used to align the sequences obtained with the corresponding sequences of their phylogenetic neighbour species by MUSCLE and to obtain phylogenetic trees by using the maximum–likelihood method and the general time–reversible model with invariant sites [Tamura et al., 2011]. The robustness of the topology of the trees was estimated by bootstrap analysis with 100 replicates [Felsenstein, 1985]. MEGA5 was also used to calculate the sequence similarity values.

Genotypic fingerprinting and DNA G+C content

Genotypic fingerprints were obtained via $(GTG)_5$–PCR, as described by Gevers [2001]. The DNA G+C content of the strains was determined as previously described [Cleenwerck et al., 2008].

Phenotypic characterization

To detect enzymes and substrate utilization of the four novel isolates and the type strain of their closest phylogenetic neighbour, *B. bohemicum* LMG 27797$^T$, API ZYM and API

50CHL strips (bioMérieux) were inoculated with dense cell suspensions (McFarland 5 and 2, respectively) which were thoroughly homogenized by vortexing. The API 50CHL strips were read after 5 days of incubation. The ability to hydrolyse gelatin was determined in MRS broth with 12% gelatin. Gram–stain behaviour, endospore staining and verification of oxidase and catalase activity were performed using standard microbiological procedures [Macfaddinn, 1980]. Growth and gas production was determined in triplicate in MRS broth (Oxoid) with inverted Durham tubes at different temperatures (10, 15, 25, 37 and 45°C) or pH values (pH 3, 5, 7 and 9). Cell morphology and motility was checked with light microscopy. Fructose 6–phosphate phosphoketolase activity and production of short chain fatty acids was determined after growth in MRS broth for 3 days as described by Orban and Patterson [2000] and De Baere et al. [2013], respectively. Production of D– and L–lactic acid was determined by using the D–/L–Lactic Acid (D–/L–Lactate) (Rapid) Assay Kit (Megazyme).

### 4.3.4 Results and Discussion

In the framework of a bumblebee gut microbiota study, four *Bifidobacterium* isolates (LMG $28292^T$, R–53560, R–53124, LMG 28626) were obtained from the gut of three different bumblebees, identified as *B. lapidarius* (LMG $28292^T$, R–53560), *B. hypnorum* (LMG 28626) and *B. terrestris* (R–53124). The MALDI–TOF MS profiles of the four isolates were distinct from those of established *Bifidobacterium* species (data not shown), but identical to each other (Figure 4.6). The $(GTG)_5$–PCR profiles of the four isolates were indistinguishable (Supplementary Figure 4.15). Activity of a fructose–6–phosphate phosphoketolase enzyme was observed for LMG $28292^T$, which is a key enzyme in the degradation of hexoses by bifidobacteria (the so called bifid shunt). Acetic and lactic acid were produced by this bifid shunt in the theoretical molar ratio of 3:2, respectively [Yin et al., 2005].

**Figure 4.6:** MALDI–TOF MS profiles of *Bifidobacterium commune* isolates (LMG $28292^T$, R–53560, R–53124, LMG 28626) and their closest relative *B. bohemicum* LMG $27797^T$. The dendrogram was derived from unweighted pair group cluster analysis (UPGMA) of the fingerprints with levels of linkage expressed as Pearson correlation coefficients.

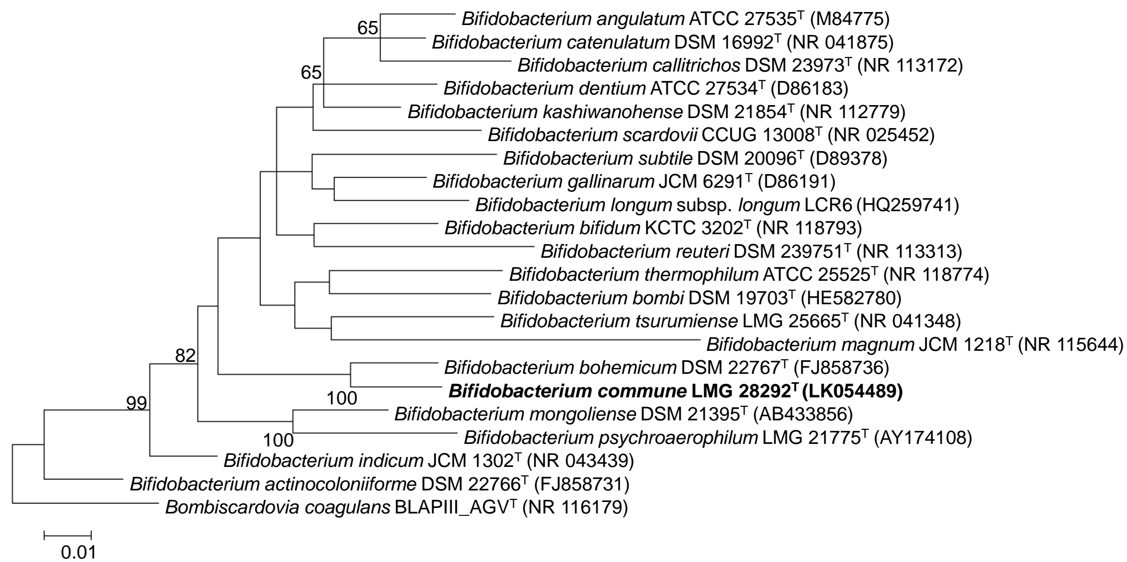



**Figure 4.7:** Restricted phylogenetic tree based on 16S rRNA gene sequences of established *Bifidobacterium* species and the *Bifidobacterium commune* type strain LMG 28292$^{T}$. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage is 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (five categories (+G, parameter = 0.1000)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 73.4797% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 22 nucleotide sequences with the *Bombiscardovia coagulans* type strain as the outgroup. All positions with less than 95%site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1286 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011]

Comparative 16S rRNA gene sequence analysis demonstrated that strain LMG 28292$^{T}$ formed a distinct lineage within the genus *Bifidobacterium*, with *B. bohemicum* as nearest neighbour species (96.8% sequence similarity towards *B. bohemicum* LMG 27797$^{T}$); this clustering was supported by a bootstrap value of 97% (Supplementary Figure 4.16). A more restricted 16S rRNA gene sequence based phylogenetic tree is shown in Figure 4.7. Protein encoding genes such as *hsp60*, which is commonly used in taxonomic and identification studies of the genus *Bifidobacterium*, evolve at a higher pace and have a discriminatory power superior to that of the more conserved 16S rRNA gene. The *hsp60* gene sequences of the four isolates were identical and comparative sequence analysis confirmed *B. bohemicum* as their nearest neighbour species with 85.8% sequence similarity towards *B. bohemicum* LMG 27797$^{T}$, although phylogenetic analysis indicated a relationship to *B. bombi* that did not receive significant bootstrap support (Supplementary Figure 4.17). Figure 4.8 shows an *hsp60*

gene sequence based phylogenetic tree comprising a more limited number of *Bifidobacterium* reference strains.

The DNA G+C content of strain LMG 28292$^T$ was determined as 54.3 mol%, which is within the range reported for the genus *Bifidobacterium* [Zhu, 2003]. This value differs from that of *B. bohemicum* LMG 27797$^T$ (51.2 mol%). Phenotypic characteristics were determined for the four isolates (LMG 28292$^T$, R–53560, R–53124, LMG 28626) as well as for *B. bohemicum* LMG 27797$^T$. The phenotypic characteristics of the four isolates were identical except that R–53124 and LMG 28626 did not produce acid from D–fructose, methyl–$\alpha$–D–glucopyranoside and sucrose, and that LMG 28626 does not produce acid from amygdalin. Strain dependent characteristics and differential characteristics between LMG 28292$^T$ and *B. bohemicum* LMG 27797$^T$ determined by the API 50CHL microtest system are shown in Table 4.6. LMG28292$^T$ and *B. bohemicum* LMG 27797$^T$ produce acid from D–ribose, D–galactose, D–glucose, D–fructose, arbutin, esculin ferric citrate, salicin, D–melibiose, potassium–5–ketogluconate (although weakly) and D–raffinose, but not from glycerol, erythritol, L–rhamnose, D–mannitol, D–sorbitol, methyl–$\alpha$–D–mannopyranoside, D–maltose, D–lactose, D–trehalose, inulin, D–melezitose, glycogen, xylitol, D–turanose, D–lyxose, D–fucose, L–fucose, D–arabitol, L–arabitol and potassium–2–ketogluconate. Only the L–isomeric form of lactic acid was found to be produced. Strain LMG 28292$^T$ and *B. bohemicum* LMG 27797$^T$ exhibit activity of leucine arylamidase, acid phosphatase, naphtol–AS–BI–phosphohydrolase, $\alpha$– and $\beta$–galactosidase and $\alpha$– and $\beta$–glucosidase. LMG 28292$^T$ and *B. bohemicum* LMG 27797$^T$ do not exhibit gelatinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, $\alpha$–chymotrypsin, $\beta$–glucuronidase, N–acetyl–$\beta$–glucosaminidase, $\alpha$–mannosidase and $\alpha$–fucosidase activity. Together, these data demonstrate that the isolates LMG 28292$^T$, R–53560, R–53124 and LMG 28626 represent a genotypically and phenotypically coherent taxon that is closely related to *B. bohemicum*, from which it can be differentiated by means of phylogenetic, genotypic and phenotypic data. The MALDI–TOF MS (Figure 4.6) and $(GTG)_5$–PCR profiles (Supplementary Figure 4.15), and the *hsp60* sequences of all four isolates were indistinguishable; however, three different phenotypes were observed among the four isolates by means of the API 50CHL microtest system. We therefore propose to classify these four isolates into the novel species *Bifidobacterium commune* sp. nov. with LMG 28292$^T$ as the type strain. Four *Bifidobacterium* species have now been isolated from the guts of bumblebee species [Killer et al., 2009, 2011]. *Bifidobacteria* are also part of the gut microbiota of other hosts and their beneficial influence on the immune system and the production of health promoting compounds such as short chain fatty acids, vitamins and antimicrobials has been well established [Picard et al., 2005]. Therefore, their potential beneficial effect to bumblebee health should be further explored.

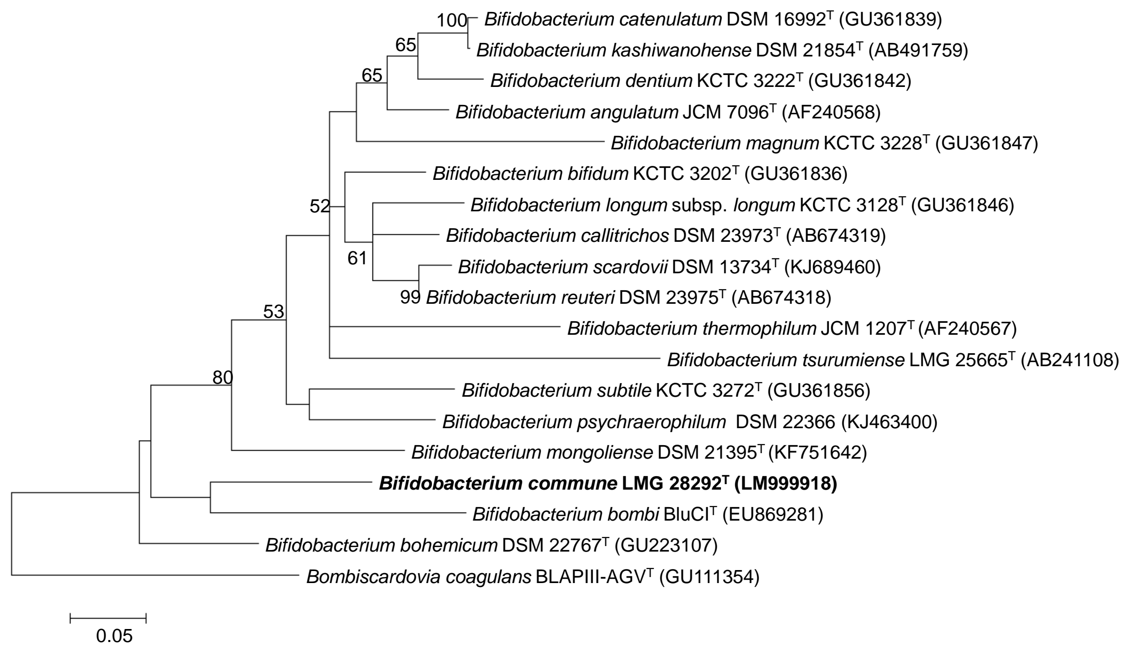



**Figure 4.8:** Restricted phylogenetic tree based on *hsp60* gene sequences of established *Bifidobacterium* species and the *Bifidobacterium commune* type strain LMG 28292$^{T}$. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage is 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites [five categories (+G, parameter = 0.2370)]. The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 52.3083% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences with the *Bombiscardovia coagulans* type strain as the outgroup. Codon positions included were 1st + 2nd + 3rd + Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 332 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].

**Table 4.6:** Differential phenotypic characteristics between *Bifidobacterium commune* LMG 28292$^T$ and *Bifidobacterium bohemicum* LMG 27797$^T$

| Phenotypic characteristics | LMG 28292$^T$ | LMG 27797$^T$ |
|---|---|---|
| Production of acid from: | | |
| D–Arabinose | – | –* |
| L–Arabinose | – | + |
| D–Xylose | – | + |
| L–Xylose | – | w |
| D–Fructose | + (2/4)[a] | + |
| D–Adonitol | – | w |
| Methyl–$\beta$–D–xylopyranoside | – | w |
| D–Mannose | – | + |
| L–Sorbose | – | w |
| Dulcitol | – | w |
| Inositol | – | w |
| Methyl–$\alpha$–D–glucopyranoside | + (2/4)a | +* |
| N–Acetylglucosamine | – | + |
| Amygdalin | + (3/4)a | +* |
| D–Cellobiose | – | w |
| Sucrose | + (2/4)a | w |
| Starch | – | + |
| Gentiobiose | – | + |
| D–Tagatose | – | w |
| Potassium gluconate | – | w |

+: positive, –: negative, w: weakly positive

*data obtained in the present study not corresponding to published data

[Killer et al., 2011].

[a] number of isolates with a reaction identical to that of the type strain.

### 4.3.5 Description of *Bifidobacterium commune* sp. nov.

*Bifidobacterium commune* (com.mu'ne. L. n. adj. *commune* common, widespread, referring to the occurrence of this species in the gut of multiple bumblebee species).

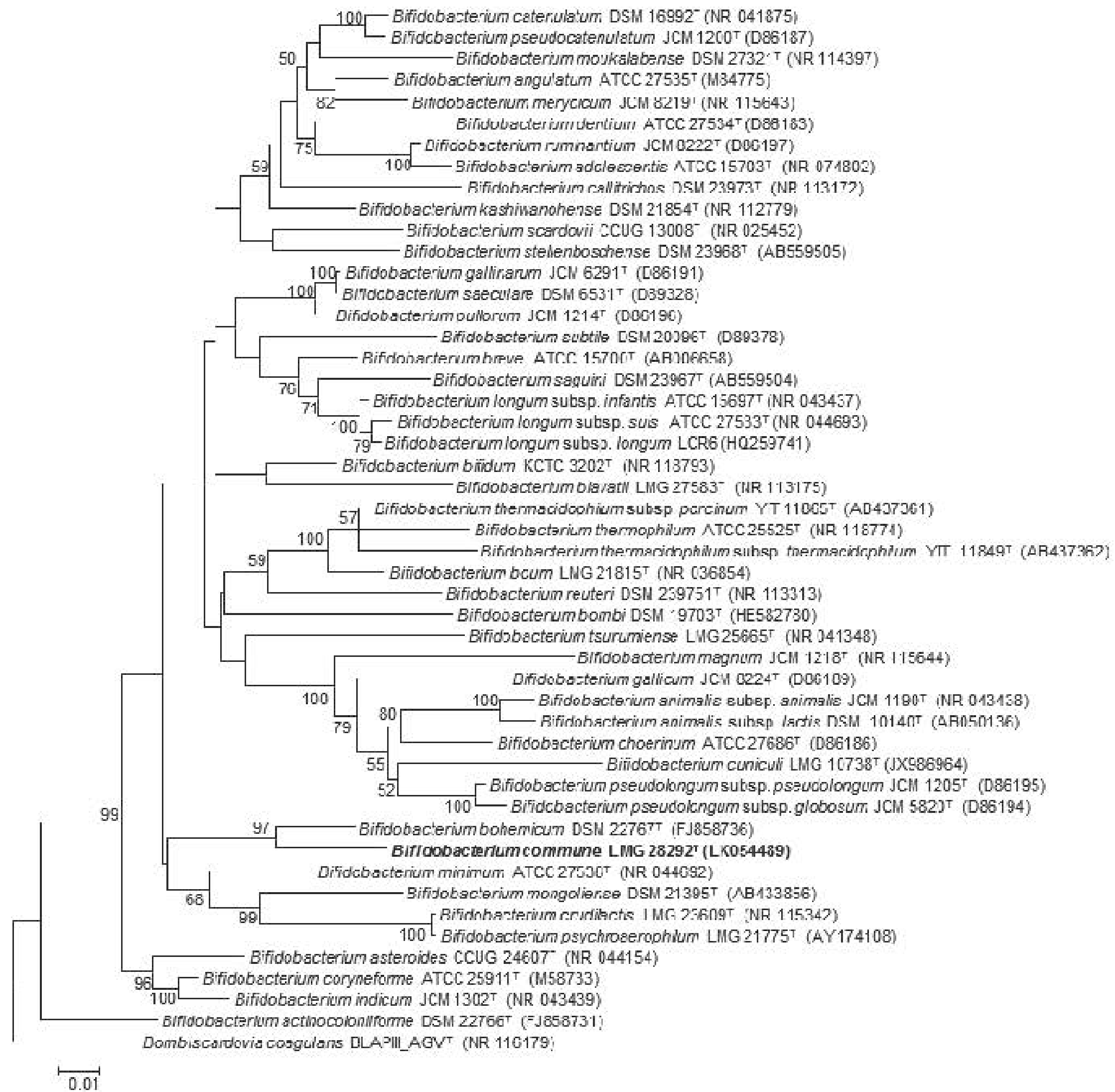
Cells are Gram–stain positive, non–motile, fructose–6–phosphate phosphoketolase positive, bifurcated and elongated cocci (0.5–1 µm wide and 2 µm long). No oxidase or catalase activity. Endospores are not produced. Growth is observed after 48 h on MTPY and MRS agar at 37°C in an anaerobic and micro–aerobic atmosphere. Colonies on MRS agar are white to beige, 1 mm, round and shiny. Growth is observed in MRS broth at 10 and 15°C (although weak), 37°C and pH 5 and pH 7 but not at 45°C, pH 3 and pH 9. No gas is produced from glucose. L–lactic acid and acetic acid are produced from glucose at a molar ratio of 2:3. Acid is produced from methyl–$\alpha$–D–glucopyranoside, amygdalin, sucrose, D–ribose, D–galactose, D–glucose, D–fructose, arbutin, esculin ferric citrate, salicin, D–melibiose, potassium–5–ketogluconate (although weak) and D–raffinose, but not from D– and L–arabinose, D– and L–xylose, D–adonitol, methyl–$\beta$–D–xylopyronoside, D–mannose, L–sorbose, dulcitol, inositol, N–acetylglucosamine, D–cellobiose, starch, gentiobiose, D–tagatose, potassium gluconate, glycerol, erythritol, L–rhamnose, D–mannitol, D–sorbitol, methyl–$\alpha$–D–mannopyranoside, D–maltose, D–lactose, D–trehalose, inulin, D–melezitose, glycogen, xylitol, D–turanose, D–lyxose, D–fucose, L–fucose, D–arabitol, L–arabitol and potassium 2–ketogluconate. Positive for leucine arylamidase, acid phosphatase, naphtol–AS–BI–phosphohydrolase, $\alpha$– and $\beta$–galactosidase and $\alpha$– and $\beta$–glucosidase activity. Negative for gelatinase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cysteine arylamidase, trypsin, $\alpha$–chymotrypsin, $\beta$–glucuronidase, N–acetyl–$\beta$–glucosaminidase, $\alpha$–mannosidase and $\alpha$–fucosidase activity. The DNA G+C content of the type strain is 54.3%. The type strain LMG 28292$^{T}$ (=DSM 28792$^{T}$), was isolated from the gut of a *Bombus lapidarius* bumblebee in 2013 in Ghent, Belgium. The accession numbers for the 16S rRNA and *hsp60* gene sequences of *B. commune* LMG 28292$^{T}$ are LK054489 and LM999918, respectively.

### 4.3.6 Acknowledgments

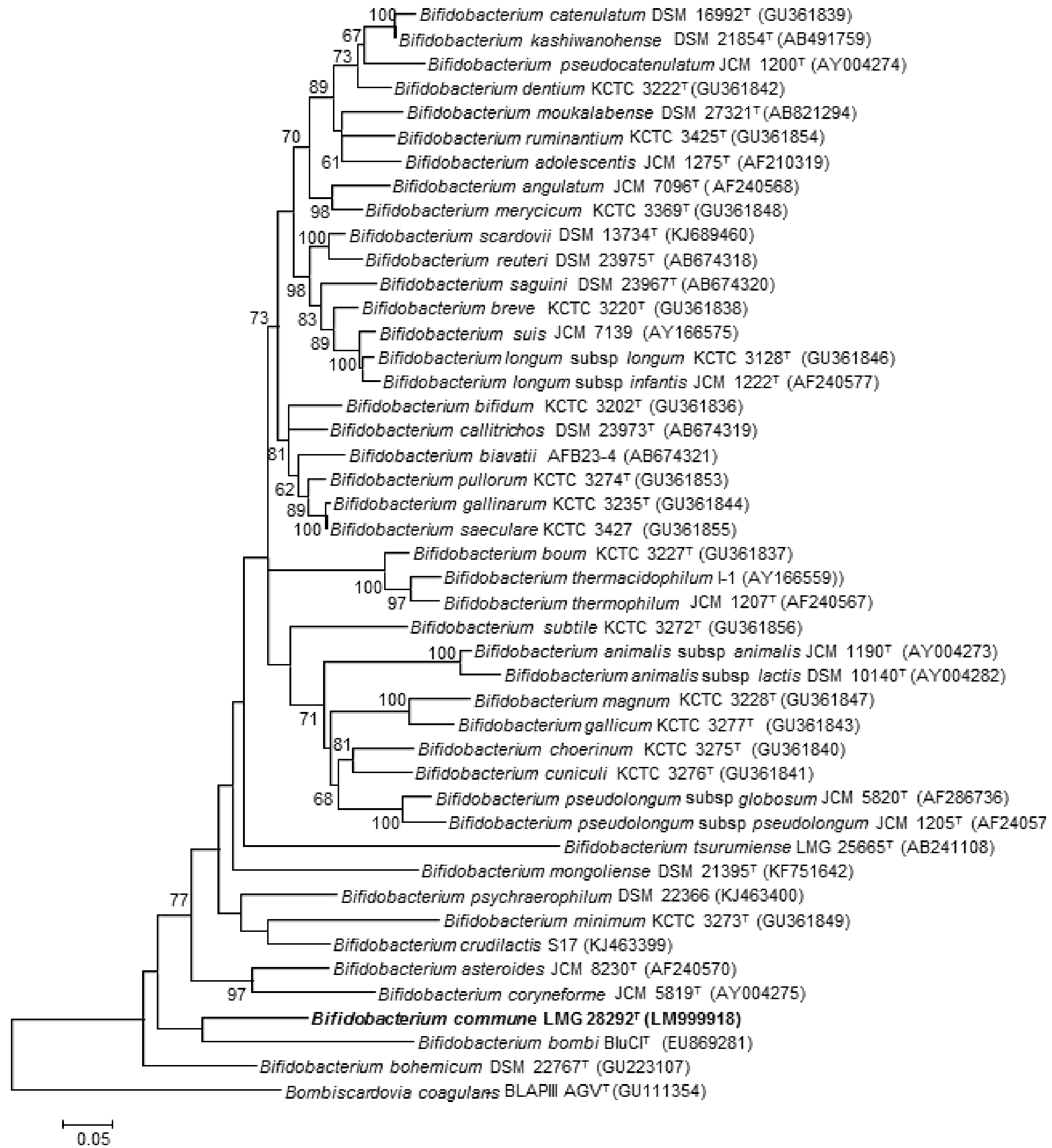
The authors acknowledge support by Ghent University-Special Research Fund (BOF). Compliance with ethical standards The present research involved sampling of bumble bees for which no permission was required as bumblebees are not included in the ''Decree of Species (het Soortenbesluit (http://codex.vlaanderen.be/Zoeken/Document.aspx?DID=1018227¶m =informatie])'' of the Flemish government with inception on 01/09/2009. The authors do not have a conflict of interest.

### 4.3.7 Supplementary material

**Supplementary Figure 4.15:** MALDI–TOF MS profiles of *Bifidobacterium commune* isolates (LMG 28292$^{T}$, R–53560, R–53124, LMG 28626) and their closest relative *B. bohemicum* LMG 27797$^{T}$. The dendrogram was derived from unweighted pair group cluster analysis (UPGMA) of the fingerprints with levels of linkage expressed as Pearson correlation coefficients.




**Supplementary Figure 4.16:** Phylogenetic tree based on 16S rRNA gene sequences of established *Bifidobacterium* species and the *Bifidobacterium commune* type strain LMG 28292$^{T}$. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000].The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage is 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1416)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 69.5197% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 49 nucleotide sequences with the *Bombiscardovia coagulans* type strain as the outgroup. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1566 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].




**Supplementary Figure 4.17:** Phylogenetic tree based on *hsp60* gene sequences of established *Bifidobacterium* species and the *Bifidobacterium commune* type strain LMG 28292$^T$. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed [Felsenstein, 1985]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage is 50% or higher [Felsenstein, 1985]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1.0225)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 49.0810% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 45 nucleotide sequences with the *Bombiscardovia coagulans* type strain as the outgroup. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 510 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [Tamura et al., 2011].

## 4.4 *Apibacter mensalis* sp. nov.: a rare member of the bumblebee gut microbiota.

**Redrafted from:** Jessy Praet, Maarten Aerts, Evie De Brandt, Ivan Meeus, Guy Smagghe, Peter Vandamme (2016). *Apibacter mensalis* sp. nov.: a rare member of the bumblebee gut microbiota. International Journal of Systematic and Evolutionary Microbiology, 66, 1645–1651.

**Author contributions:** JP and PV conceived the study and wrote the manuscript. JP, MA and EDB performed the experiments. IM and GS proofread the manuscript.

### 4.4.1 Abstract

Isolates LMG 28357$^T$ (=R–53146$^T$) and LMG 28623 were obtained from gut samples of *Bombus lapidarius* bumblebees caught in Ghent, Belgium. They had identical 16S rRNA gene sequences which were 95.7% identical to that of *Apibacter adventoris* wkB301$^T$, a member of the family *Flavobacteriaceae*. Both isolates had highly similar matrix–assisted laser desorption/ionization time–of–flight (MALDI–TOF) MS and randomly amplified polymorphic DNA (RAPD) profiles. A draft genome sequence was obtained for strain LMG 28357$^T$ (Gold ID Gp0108260); its DNA G+C content was 30.4%, which is within the range reported for members of the family *Flavobacteriaceae* (27 to 56 mol%) and which is similar to that of the type strain of *A. adventoris* (29.0 mol%). Whole–cell fatty acid methyl ester analysis of strain LMG 28357$^T$ revealed many branched-chain fatty acids, a typical characteristic of bacteria of the family *Flavobacteriaceae* and a profile that was similar to that reported for *A. adventoris* wkB301$^T$. MK6 was the major respiratory quinone, again conforming to bacteria of the family *Flavobacteriaceae*. The isolates LMG 28357$^T$ and LMG 28623 could be distinguished from *A. adventoris* strains through their oxidase activity. On the basis of phylogenetic, genotypic and phenotypic data, we propose to classify both isolates as representatives of a novel species of the genus *Apibacter*, *Apibacter mensalis* sp. nov., with LMG 28357$^T$ (=DSM 100903$^T$ =R–53146$^T$) as the type strain.

### 4.4.2 Introduction

Insect pollination is of major importance for global food production with a total economic value of €153 billion in 2005 [Gallai et al., 2009]. In Europe, 84% of the cultivated crops depend directly on insect pollination [Gallai et al., 2009] to which especially bumblebees, honeybees and solitary bees contribute. Bumblebees have experienced distribution declines in various parts of the world, with many species living in restricted areas and some critically endangered on a larger scale (http://www.iucnredlist.org). These declines, presumably caused by a combination of factors such as pesticide use, pathogen emergence and changes in climate and agricultural practices [Goulson et al., 2008b; Meeus et al., 2011] may have a detrimental impact on agriculture and ecosystem integrity [Klein et al., 2007]. To reverse or avoid further declines, governments have stimulated farmers to improve the agricultural landscape and environment for bees (http://www.ec.europa.eu). In addition, the importance of the bumblebee gut microbiota for bee health has been established [Cariveau et al., 2014; Koch and Schmid-Hempel, 2011b, 2012]. The cultivable microbiota of these insects is gradually being characterized [Killer et al., 2009, 2010a, 2011; Kwong and Moran, 2013; Praet et al., 2015] and is a first step towards understanding of their functionality with regard to their host, environment and/or associated microbiota.

The gut microbiota of bumblebees resembles that of honeybees and often comprises different species of the same genus [Koch and Schmid-Hempel, 2011b]. It consists of a core set of genera belonging to the families *Neisseriaceae* (*Snodgrassella*), *Orbaceae* (*Gilliamella*), *Lactobacillaceae* (*Lactobacillus*) and *Bifidobacteriaceae* (*Bifidobacterium*) and several non–core bacteria. Koch and Schmid-Hempel [2011a] reported the presence of a bumblebee gut phylotype belonging to the phylum Bacteroidetes which has only sporadically been detected in bumblebees and therefore its prevalence is presumably low [Koch and Schmid-Hempel, 2011b; Meeus et al., 2015]. Recently, three isolates from *Apis dorsata* and *Apis cerana* gut samples that represented this phylotype were formally classified as representatives of *Apibacter adventoris* [Kwong and Moran, 2016b]. In the present study, we present a polyphasic taxonomic characterization of two bumblebee gut isolates representing the same Bacteroidetes phylotype and propose to classify them as representatives of a novel species of the genus *Apibacter*.

### 4.4.3 Material and methods, Results and Discussion

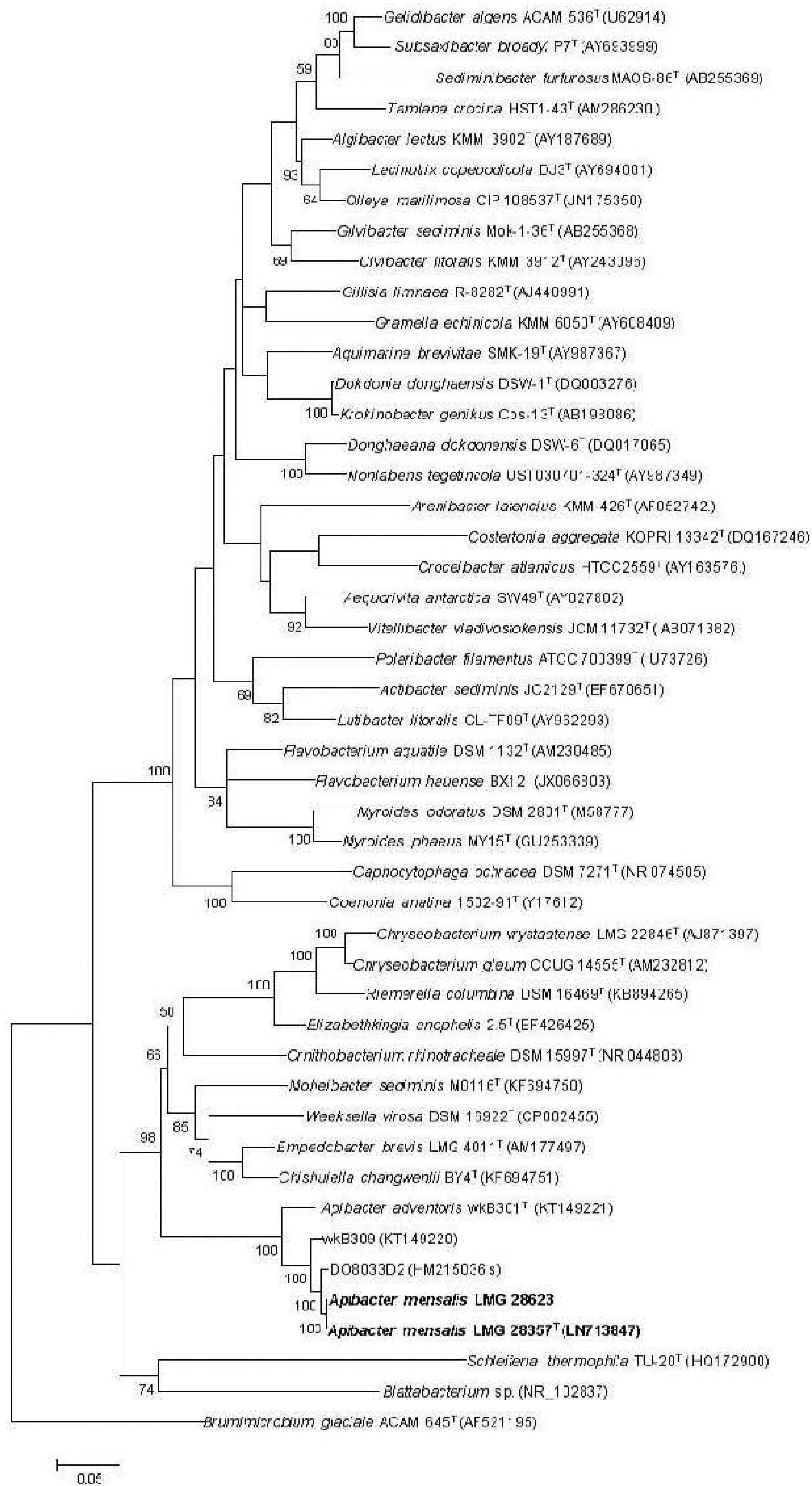
In August 2013, *Bombus lapidarius* bumblebees were caught in the field in the region of Ghent, Belgium, and identified by their colour pattern. The bumblebees were immobilized at –20°C for 10 min and surface sterilized with 2.5% Umonium[38] Master (Laboratoire Huckert's International) before dissecting out their crop and gut. The crops and guts were homogenized in 250 µl saline solution (0.1% peptone, 0.1% Tween 80, 0.85% NaCl, 5% DMSO) with a sterile micro–pestle. Subsequently, the cell suspensions were serially diluted to $10^{-4}$ in physiological saline (0.85% NaCl), plated on all culture (AC) agar (Sigma–Aldrich) and incubated micro–aerobically at 37°C. After 5 days, colonies were picked up from the agar plates and third–

generation axenic isolates were dereplicated by matrix–assisted laser desorption/ionization time–of–flight (MALDI–TOF) MS followed by curve–based data analysis [Ghyselinck et al., 2011] using BioNumerics 5.1 software (Applied Maths). Representative isolates of each MALDI–TOF MS cluster were selected for further identification. The MALDI–TOF MS profiles of two isolates (LMG 28357$^T$ and LMG 28623) from two different *B. lapidarius* bumblebees sampled in Bourgoyen in Ghent, Belgium, were highly similar (Supplementary Figure 4.18) and clustered separately from those of all other isolates in our database (data not shown).

Both isolates were routinely grown micro–aerobically on AC agar at 37°C. Random amplified polymorphic DNA (RAPD) analysis was performed on both isolates with primer RAPD–272 as described by [Williams et al., 1990]. The RAPD profiles obtained were highly similar and differed only in the intensity of some bands (Supplementary Figure 4.19).

To identify both isolates their 16S rRNA gene sequences were determined as previously described [De Bruyne et al., 2007] and were identical. MEGA6 analysis designated *A. adventoris* as the most closely related neighbour taxon with a validly published name, yet only 95.7% 16S rRNA gene sequence identity was calculated towards its type strain, wkB301$^T$ [Kwong and Moran, 2016b]. The 16S rRNA gene sequence of isolates LMG 28357$^T$ and LMG 28623 was also 98.4% similar to that of a second *A. adventoris* strain, wkB309, highlighting a considerable genetic divergence within the latter species. Based on the threshold of 98.65% 16S rRNA gene sequence identity for species differentiation, the isolates LMG 28357$^T$ and LMG 28623 represent a novel species of the genus *Apibacter* [Kim et al., 2014]. A 16S rRNA gene sequence based phylogenetic tree comprising the isolates LMG 28357$^T$ and LMG 28623 and their closest neighbours based on an EzTaxon–e analysis is shown in Figure 4.9.

Whole genomic DNA of isolate LMG 28357$^T$ was obtained as described by Pitcher et al. [1989]. A draft genome sequence (Gold ID Gp0108260) was generated at the DOE Joint Genome Institute (JGI) using Illumina technology. An Illumina std shotgun library was constructed and sequenced using the Illumina HiSeq 2000 platform. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. Raw Illumina sequence data were passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artefacts. The following steps were then performed for assembly: filtered Illumina reads were assembled using Velvet (version 1.2.07; [Zerbino and Birney, 2008]), 1–3 kb simulated paired–end reads were created from Velvet contigs using wgsim (version 0.3.0; https://github.com/lh3/wgsim) and Illumina reads were assembled with simulated read pairs using Allpaths–LG (version r46652; [Gnerre and MacCallum, 2011]). Genome annotation was performed by RAST [Aziz et al., 2008]. The genome size of strain LMG 28357$^T$ was 2.3 Mb and consisted of 2095 coding sequences. Its DNA G+C content was determined by using GC calculator (http://www.genomicsplace.com/gc_calc.html) and was 30.4 mol%. This conforms to values reported for members of the family *Flavobacteriaceae* (27 to 56 mol%) and is similar to the value of the type strain of *A. adventoris* (29.0 mol%) [Bernardet, 2010].



Gelidibacter algens ACAM 536$^T$ (U62914)
Sediminibacter furfurosus MAOS-86$^T$ (AB255369)
Tamlana crocina HST1-43$^T$ (AM286230)
Algibacter lectus KMM 3902$^T$ (AY187689)
Olleya marilimosa CIP 108537$^T$ (JN175350)
Gilvibacter sediminis Mok-1-36$^T$ (AB255368)
Gillisia limnaea R-8282$^T$ (AJ440991)
Gramella echinicola KMM 6050$^T$ (AY608409)
Aquimarina brevivitae SMK-19$^T$ (AY987367)
Dokdonia donghaensis DSW-1$^T$ (DQ003276)
Krokinobacter genikus Cos-13$^T$ (AB198086)
Donghaeana dokdonensis DSW-6$^T$ (DQ017065)
Costertonia aggregata KOPRI 13342$^T$ (DQ167246)
Aequorivita antarctica SW49$^T$ (AY027802)
Vitellibacter vladivostokensis JCM 11732$^T$ (AB071382)
Polaribacter filamentus ATCC 700399$^T$ (U73726)
Actibacter sediminis JC2129$^T$ (EF670651)
Flavobacterium aquatile DSM 1132$^T$ (AM230485)
Flavobacterium hauense BX12 (JX066303)
Myroides odoratus DSM 2801$^T$ (M58777)
Myroides phaeus MY15$^T$ (GU253339)
Capnocytophaga ochracea DSM 7271$^T$ (NR 074505)
Coenonia anatina 1502-91$^T$ (Y17612)
Chryseobacterium gleum CCUG 14555$^T$ (AM232812)
Riemerella columbina DSM 16469$^T$ (KB894265)
Elizabethkingia anophelis 2.5$^T$ (EF426425)
Ornithobacterium rhinotracheale DSM 15997$^T$ (NR 044803)
Moheibacter sediminis M0116$^T$ (KF394750)
Weeksella virosa DSM 16922$^T$ (CP002455)
Empedobacter brevis LMG 4011$^T$ (AM177497)
Chishuiella changwenlii BY4$^T$ (KF694751)
Apibacter adventoris wkB301$^T$ (KT149221)
wkB309 (KT149220)
DO8033D2 (HM215036 s)
**Apibacter mensalis LMG 28623**
**Apibacter mensalis LMG 28357$^T$ (LN713847)**
Schleiferia thermophila TU-20$^T$ (HQ172900)
Blattabacterium sp. (NR_102837)
Brumimicrobium glaciale ACAM 645$^T$ (AF521195)
100
59
93
64
69
100
100
92
69
82
100
84
100
100
100
100
100
50
66
98
85
74
100
100
100
100
100
74
0.05

**Figure 4.9:** Phylogenetic tree based on 16S rRNA gene sequences of isolates LMG 28357$^T$ and LMG 28623 and their closest neighbours. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log–likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; +G, parameter= 0.7623). The rate variation model allowed for some sites to be evolutionarily invariable (+I, 55.1926% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 47 nucleotide sequences. All positions with less than 95% site coverage were eliminated. There were a total of 1334 positions in the final dataset.

The genome statistics of strain LMG 28357$^T$ are summarized in Table 4.7. Most annotated coding sequences belonged to the following RAST subsystems [Aziz et al., 2008]: cofactors, vitamins, prosthetic groups, pigments (12%); protein metabolism (12%); amino acids and derivatives (17%); and carbohydrates (10%). Complete [riboflavin (=vitamin B2) and lipoic acid] or nearly complete [pyridoxal 5–phosphate (=vitamin B6), menaquinone 6 (=vitamin K2), vitamin B12, niacin (=vitamin B3) and folate] biosynthesis pathways of several vitamins are present. These vitamins may provide extra nutrients for bumblebees that feed on pollen, which is difficult to digest and often depleted in fat–soluble vitamins such as vitamins K, D and E [Schmidt, 1997]. Protein and amino acid metabolism are also two abundant subsystems with only a minority of genes involved in protein degradation (11%) and amino acid degradation (13%). The biosynthesis pathways for phenylalanine and tryptophan, two essential amino acids for bumblebees which are sometimes lacking in pollen [Roulston and Cane, 2000], could be reconstructed completely. The assigned functions of the genes of the vitamin and amino acid production pathways were checked with InterProScan, and the position of the proteins in the cell was checked with PSORT and TMpred. Tblastn was used to search for missing genes by aligning the translated genome to homologous protein sequences of a close relative.
Genomes of obligate or early symbionts are often very small and contain few coding sequences [McCutcheon and Moran, 2012]. Genes involved in replication, transcription, translation and serving the host are relatively dominant in such genomes while other functions are in the process of being lost. Although the genome of strain LMG 28357$^T$ is relatively small, degeneracy was not observed.

**Table 4.7:** Genome statistics of *A. mensalis* sp. nov. LMG 28357$^T$

| | LMG 28357$^T$ |
|---|---|
| Number of scaffolds | 42 |
| Size (Mb) | 2.3 |
| DNA G+C content | 30.4 |
| Scaffold N/L50 | 5/183.1 kb |
| Read coverage | 302X |
| Number of CDS calls | 2095 |
| Number of tRNA calls | 38 |
| Number of rRNA calls | 7 |

To obtain a higher phylogenetic resolution, a phylogenetic tree based on 20 protein sequences of isolate LMG 28357$^T$ and its closest neighbours was reconstructed (Figure 4.10). The 20 protein sequences (encoded by genes *alaS*, *atpA*, *dnaA*, *dnaN*, *ftsZ*, *fusA*, *groEL*, *gyrB*, *lepA*, *metK*, *nusG*, *pfkA*, *pyrG*, *recA*, *rplA*, *rplB*, *rpoB*, *rpsB*, *sdhA* and *secA*) were obtained from the genome sequence of strain LMG 28357$^T$ and from its closest relatives to produce a tree based on 10444 positions. The MEGA6 software package was used to align the sequences by MUSCLE and to obtain phylogenetic trees by using the maximum–likelihood method and the general time–reversible model with invariant sites for the 16S rRNA gene based tree [Tamura et al., 2013b] and the JTT matrix–based model for the protein sequences–based tree. The robustness of the topology of the trees was estimated by bootstrap analysis with 100 replicates [Felsenstein, 1985].

Biochemical characteristics were determined for isolates LMG 28357$^T$ and LMG 28623 and compared with those reported for *A. adventoris* (Table 4.8). To test substrate utilization and enzyme activity, API 20NE and API ZYM galleries (bioMérieux) were inoculated with dense cell suspensions (McFarland 0.5). The API 20NE strips were read after 2 days of incubation at 37°C. Gram–stain–reaction, and verification of oxidase, catalase and DNase activity and hydrolysis of Tween 60, Tween 80, starch, casein and gelatin were performed using standard microbiological procedures [Macfaddinn, 1980]. Lactose fermentation was tested on MacConkey agar. Growth was determined on AC agar at different temperatures (20, 28, 37 and 45 °C) and in AC broth at different pH values (pH 3, 5, 7 and 9) and NaCl concentrations (5, 6, 7 and 8%). Growth was also tested on AC agar and Columbia blood agar (Oxoid) at 37°C in aerobic, micro–aerobic (80% $N_2$, 15% $CO_2$ and 5% $O_2$) and anaerobic (80% $N_2$, 10% $H_2$, 10% $CO_2$) atmospheres. Cell morphology and motility was checked with phase–contrast microscopy (BX40F 4; Olympus). Both isolates could be differentiated from the type strain of *A. adventoris* by means of their oxidase activity and ability to grow anaerobically and aerobically; they also differed from strain wkB309 by means of their oxidase and nitrate reduction activity, ability to grow anaerobically and aerobically, and lack of motility and $\beta$–galactosidase activity (Table 4.8).

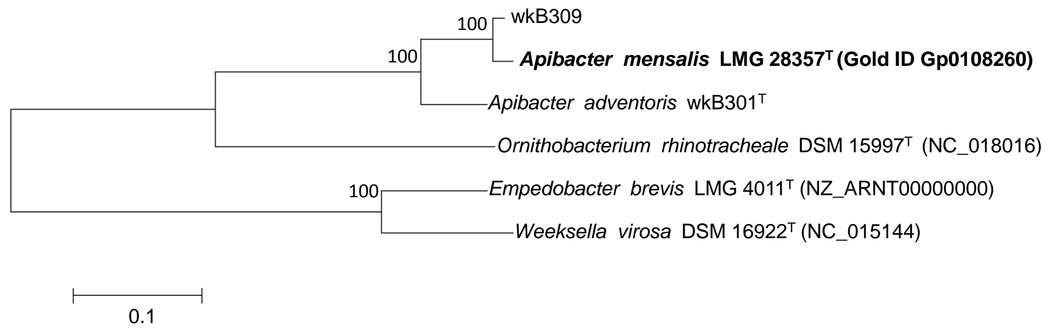



**Figure 4.10:** Phylogenetic tree based on the alignment of 20 concatenated protein sequences from strain LMG 28357$^T$ and its closest neighbours based on EzTaxon–e. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour–Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log–likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories; + G, parameter= 0.6013). The rate variation model allowed for some sites to be evolutionarily invariable (+I, 11.1008% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved six amino acid sequences. All positions with less than 95% site coverage were eliminated. There were a total of 10 444 positions in the final dataset.

Fatty acid methyl ester (FAME) analysis was performed for strain LMG 28357$^T$ using an Agilent Technologies 6890N gas chromatograph. Cultivation of the strain and extraction of the fatty acids were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI), except that fatty acids were extracted from a culture grown on AC agar at 37°C under micro-aerobic conditions for 48h. The peaks of the profiles were identified using the TSBA50 identification library version 5.0 (MIDI). FAME analysis revealed iso–$C_{17:0}$ 3-OH (24.25%), iso–$C_{15:0}$ (16.92%) and $C_{16:0}$ (14.70%) as the predominant fatty acids while several other fatty acids were present in lesser amounts. The fatty acids iso–$C_{17:0}$ 3–OH (18.0%), iso–$C_{15:0}$ (23.2%) and $C_{16:0}$ (15.1%) were also the major fatty acids reported in the type strain of *A. adventoris* [Kwong and Moran, 2016b].

**Table 4.8:** Differential phenotypic characteristics of strain LMG 28357$^T$ and *A. adventoris* wkB301$^T$ and wkB309.

The phenotypic characteristics of both *A. mensalis* sp. nov. isolates (LMG 28357$^T$ and LMG 28623) were identical. Data for *A. adventoris* wkB301$^T$ and wkB309 from Kwong and Moran [2016b].+, Positive; –, negative. All strains were positive for $\beta$–glucosidase (aesculin) and catalase activity. No growth on MacConkey agar or nutrient agar. No arginine dihydrolase, tryptophanase or urease activity, no hydrolysis of gelatin and no assimilation of D–glucose, L–arabinose, D–mannose, D–mannitol, N–acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid.

| Phenotypic characteristic | LMG 28357$^T$ | wkB301$^T$ | wkB309 |
|---|---|---|---|
| Enzymatic activity | | | |
| $\beta$–galactosidase (PNPG) | – | – | + |
| Oxidase | + | – | – |
| Nitrate reduction | + | + | – |
| Motility | – | – | + |
| Growth | micro–aerobic, aerobic (weakly) and anaerobic | micro–aerobic | micro–aerobic |
| Genome size | 2.3 Mb | 2.7 Mb | 2.3 Mb |
| DNA G+C content | 30.4 | 29.0 | 30.6 |

The respiratory quinone composition was determined for strain LMG 28357$^T$ using the protocol described by da Costa et al. [2011]. To improve the efficacy of the quinone extraction, the hexane and methanol phases were allowed to separate for 2 h at –80°C. An XBridge BEH phenyl column (pore diameter 130 Å, particle size 5 µm, 4.6 mm width x 250 mm length) was used to separate the respiratory quinones via HPLC. The samples were eluted with 100% methanol at a rate of 1 min ml$^{-1}$ and the quinones were detected at 269 nm. *Flavobacterium granuli* LMG 23365$^T$, which contains MK6, was used as a control strain [Aslam et al., 2005]. The major respiratory quinone of strain LMG 28357$^T$ was MK6, which is a typical characteristic of bacteria of the family *Flavobacteriaceae* in which MK6 is either the only or major respiratory quinone [Bernardet and Nakagawa, 2006].

Based on the phylogenetic, genotypic and phenotypic data, we propose to classify isolates LMG 28357$^T$ and LMG 28623 as representatives of a novel species of the genus *Apibacter*, *Apibacter mensalis* sp. nov., with LMG 28357$^T$ (=DSM 100903$^T$) as the type strain. Members of this novel species and their close relatives have only recently been detected in bumblebees and honeybees and with a low prevalence. Also, in our study, only two isolates were obtained from the 67 bumblebees investigated (data not shown). The 16S rRNA gene sequences of both isolates revealed 99.4 and 99.3% sequence identity to those of uncultivated clones (HM215036 and HM215037, respectively) obtained from *Bombus terrestris* and *Bombus hortorum* bumblebees [Koch and Schmid-Hempel, 2011b] suggesting that this species may not be restricted to *B. lapidarius*.

### 4.4.4 Description of *Apibacter mensalis* sp. nov.

*Apibacter mensalis* (men.sa'lis. L. adj. *mensalis* of a table; because this gut bacterium and its host are using the same nutrients and thus share the same 'dinner table').

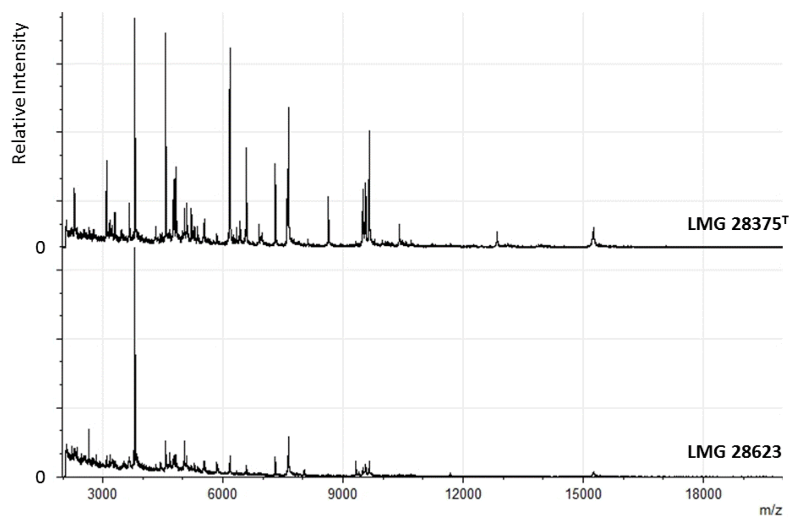
Cells are Gram-stain–negative, non–motile, rod–shaped, slightly curved and 1 µm wide and 3 to 5 µm long. Colonies are 1 mm, round, shiny and colourless after 2 days of growth on AC agar. Growth is observed after 2 days when incubated micro-aerobically and anaerobically at 37°C on AC agar and Columbia blood agar. No growth is observed at 20, 28 or 45°C. Weak growth is observed in the presence of 5 to 8% NaCl, when grown aerobically or on AC agar at pH 3 and 5. Grows at pH 7 but not at pH 9. No growth is observed on MacConkey agar or on the basal medium used to test Tween 60 and Tween 80 hydrolysis. Catalase and oxidase activities are present. Hydrolyses aesculin but not gelatin, starch or casein. Activity of alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphthol–AS–BI–phosphohydrolase and $\beta$–glucosidase is detected. Ferments glucose and reduces nitrate to nitrite. No production of lipase (C14), $\alpha$–chymotrypsin, $\alpha$– or $\beta$–galactosidase, $\beta$–glucuronidase, $\alpha$–glucosidase, N–acetyl–$\beta$–glucosaminidase, $\alpha$–mannosidase, $\alpha$–fucosidase, urease, arginine dihydrolase, gelatinase, tryptophanase, casease, amylase or DNase. No assimilation of D–glucose, L–arabinose, D–mannose, D–mannitol, N–acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate or phenylacetic acid.

The type strain contains iso–$C_{17:0}$ 3–OH, iso–$C_{15:0}$ and $C_{16:0}$ as the predominant fatty acids while several other fatty acids are present in lesser amounts: $C_{14:0}$, iso–$C_{15:0}$ 3–OH, anteiso–$C_{15:0}$, $C_{16:0}$ 2–OH, $C_{16:0}$ 3–OH, iso–$C_{17:0}$, $C_{18:0}$, $C_{18:1}$ $\omega$9c, summed feature 2 (iso–$C_{16:1}$ and/or $C_{14:0}$ 3–OH), summed feature 3 ($C_{16:1}\omega$7c and/or iso–$C_{15:0}$ 2–OH) and summed feature 5 ($C_{18:2}\omega$6,9c and/or anteiso–$C_{18:0}$). The major respiratory quinone is MK6. The type strain, LMG 28357$^{T}$ (=DSM 100903$^{T}$ =R–53146$^{T}$), was isolated from the gut of a *B. lapidarius* bumblebee sampled in 2013 in Ghent, Belgium. Its DNA G+C content is 30.4 mol%.

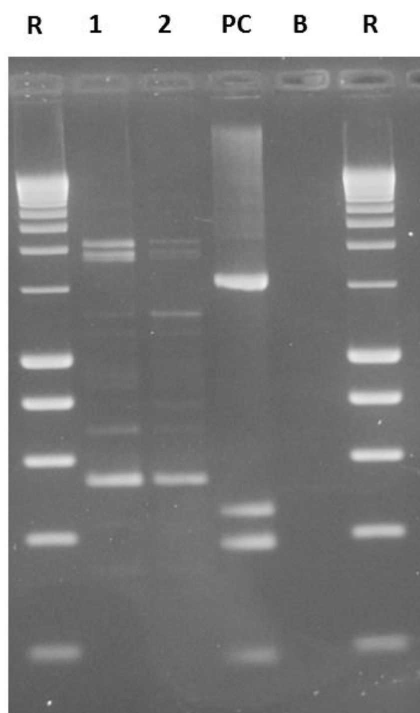
### 4.4.5 Acknowledgements

The authors acknowledge support by Ghent University–Special Research Fund (BOF). The genome sequence data were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) in collaboration with the user community. The work conducted by the US Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the US Department of Energy under contract no. DE–AC02–05CH11231.

### 4.4.6 Supplementary material




**Supplementary Figure 4.18:** Comparison of MALDI–TOF MS profiles of *Apibacter mensalis* sp. nov. LMG 28357$^{T}$ and LMG 28623 using the mMass 5.5.0 software [Strohalm et al., 2010].




**Supplementary Figure 4.19:** RAPD profiles of the two *A. mensalis* isolates LMG 28357$^{T}$ and LMG 28623. R = reference marker, 1 = LMG 28357$^{T}$, 2 = LMG 28623, PC = positive DNA control, B = blanc.

## 4.5 *Gilliamella intestini* sp. nov., *Gilliamella bombicola* sp. nov., *Gilliamella bombi* sp. nov. and *Gilliamella mensalis* sp. nov.: four novel *Gilliamella* species isolated from the bumblebee gut

**Redrafted from:** Jessy Praet, Margo Cnockaert, Ivan Meeus, Guy Smagghe, Peter Vandamme (2017). *Gilliamella intestini* sp. nov., *Gilliamella bombicola* sp. nov., *Gilliamella bombi* sp. nov. and *Gilliamella mensalis* sp. nov.: four novel *Gilliamella* species isolated from the bumblebee gut. Systematic and Applied Microbiology, 40 (4), 199-204.

**Author contributions:** JP and PV conceived the study and wrote the manuscript. JP and MC performed the experiments. IM and GS proofread the manuscript.

### 4.5.1 Abstract

Spectra of five isolates (LMG 28358$^{T}$, LMG 29879$^{T}$, LMG 29880$^{T}$, LMG 28359$^{T}$ and R-53705) obtained from gut samples of wild bumblebees of *Bombus pascuorum*, *Bombus lapidarius* and *Bombus terrestris* were grouped into four matrix–assisted laser desorption/ionisation time–of–flight mass spectrometry (MALDI–TOF MS) clusters. Random amplified polymorphic DNA analysis revealed an identical DNA fingerprint for LMG 28359$^{T}$ and R-53705 which also grouped in the same MALDI-TOF MS cluster, while different DNA fingerprints were obtained for the other isolates. Comparative 16S rRNA gene sequence analysis of the different strains LMG 28358$^{T}$, LMG 29879$^{T}$, LMG 29880$^{T}$ and LMG 28359$^{T}$ identified *Gilliamella apicola* NCIMB 14804$^{T}$ as nearest neighbour species. Average nucleotide identity values of draft genome sequences of the four isolates and of *G. apicola* NCIMB 14804$^{T}$ were below the 96% threshold value for species delineation and all four strains and *G. apicola* NCIMB 14804$^{T}$ were phenotypically distinct. The genomic DNA G+C content of LMG 28358$^{T}$, LMG 29879$^{T}$, LMG 29880$^{T}$ and LMG 28359$^{T}$ is 34.6%, 34.6%, 35.5% and 35.9%, respectively, which is comparable to that of *G. apicola* NCIMB 14804$^{T}$ (33.6%). Together, the draft genome sequences and phylogenetic and phenotypic data indicate that the four strains represent four novel *Gilliamella* species for which we propose the names *Gilliamella intestini* sp. nov., with LMG 28358$^{T}$ as the type strain, *Gilliamella bombicola* sp. nov., with LMG 28359$^{T}$ as the type strain, *Gilliamella bombi* sp. nov., with LMG 29879$^{T}$ as the type strain and *Gilliamella mensalis* sp. nov., with LMG 29880$^{T}$ as the type strain.

### 4.5.2 Introduction

Like many other pollinating insects, bumblebees are in a worldwide decline. Several studies highlighted the importance of the gut microbiota of honeybees and bumblebees in maintaining bee health [Engel et al., 2012; Koch and Schmid-Hempel, 2011a]. The gut microbiota composition of honeybees and bumblebees is similar and consists of a specific and consistent set of bacteria of which a gammaproteobacterium (referred to as Gamma–1), a betaproteobacterium (referred to as Beta) and a firmicutes (referred to as Firm–5) phylotype occur in most bee individuals [Koch and Schmid-Hempel, 2011b; Martinson et al., 2011; Meeus et al., 2015]. Some isolates belonging to each of these three phylotypes have already been characterized and have been formally named *Gilliamella apicola*, *Snodgrassella alvi* and *Lactobacillus bombicola*, respectively [Kwong and Moran, 2013; Praet et al., 2015]. Some Gamma–1 phylotype bacteria are able to degrade pectin which is a compound of the pollen cell wall and ferment mannose, arabinose, xylose and rhamnose which are sugars present in nectar and which are toxic to bees [Engel et al., 2012; Zheng et al., 2016]. This phylotype is also negatively associated with the presence of the bumblebee trypanosome parasite *Crithidia bombi* [Cariveau et al., 2014]. Since the Gamma–1 phylotype is both phylogenetically as well as functionally diverse, it has been suggested that it might comprise multiple taxa [Engel et al., 2012; Kwong and Moran, 2013]. The latter was confirmed in the present study, which reports the isolation of four novel *Gilliamella* species from gut samples of three wild bumblebee species.

### 4.5.3 Material and methods

Sampling of wild bumblebees and preparation of cell suspensions

Bumblebees were caught in the field near the city of Ghent, Belgium, and identified as *Bombus pascuorum*, *Bombus lapidarius* and *Bombus terrestris* by their colour pattern. Identifications as *B. lapidarius* and *B. terrestris* were confirmed through cytochrome oxidase I (COI) gene sequence analysis as the colour pattern of these species can be confused with that of some other *Bombus* species [Carolan et al., 2012]. The COI gene was amplified as described by Carolan et al. [2012] after Chelex® 100 resin (Bio–Rad) DNA–extraction from two bumblebee legs which were ground with a micropestle [Walsh et al., 1991]. The bumblebees were immobilized at –20°C for 10 minutes and surface sterilized with 2.5% Umonium38® Master (Laboratoire Huckert's International, Brussels, Belgium) before dissecting out their crop and gut. The crops and guts were homogenized in 125 µl saline solution (0.1% peptone, 0.1% Tween 80, 0.85% NaCl) with a sterile micro–pestle. Afterwards, 125 µl of a 10% DMSO solution was added to the cell suspensions which were stored at –80°C until further use.

Isolation of bumblebee gut bacteria and dereplication

Cell suspensions were serially diluted to $10^{-4}$ in physiological saline (0.85% NaCl), plated on M144 (23 g/l special peptone, 1 g/l soluble starch, 5 g/l NaCl, 0.3 g/l cysteine hydrochloride,

5 g/l glucose and 15 g/l agar) and all culture (AC) agar (Sigma–Aldrich) and incubated anaerobically (80% $N_2$, 10% $H_2$, 10% $CO_2$) for M144 agar or micro–aerobically (80% $N_2$, 15% $CO_2$ and 5% $O_2$) for AC agar at 37°C. After five days of incubation, colonies were picked up from the agar plates and third generation axenic subcultures were dereplicated by MALDI–TOF MS followed by curve–based spectrum analysis [Ghyselinck et al., 2011] using the BioNumerics 5.1 software (Applied Maths, Sint–Martens–Latem, Belgium).

Phylogenetic analysis

Representative isolates of each MALDI–TOF MS cluster were selected for further identification. The 16S rRNA gene sequences were determined as previously described [De Bruyne et al., 2007] and EzTaxon–e [Kim et al., 2014] was used as a first step in the identification process. The MEGA6 software package was used to align the sequences obtained with the corresponding sequence of their phylogenetic neighbour species by MUSCLE and to obtain phylogenetic trees by using the maximum–likelihood method and the general time–reversible model with invariant sites [Tamura et al., 2013a]. The robustness of the tree topologies was estimated by bootstrap analysis with 1000 replicates [Felsenstein, 1985]. MEGA6 was also used to calculate the sequence similarity levels.

RAPD fingerprinting

Random amplified polymorphic DNA (RAPD) analysis using primer RAPD–272 as described by Williams et al. [1990] was performed on five isolates tentatively identified by 16S rRNA gene sequence analysis as *Gilliamella* sp.

Draft genome sequence analysis

The genomes of strains LMG 28358$^T$ and LMG 28359$^T$ were sequenced using the Illumina HiSeq 2000 platform. All general aspects of library construction and sequencing performed at the JGI can be found at http://www.jgi.doe.gov. Raw Illumina sequence data was passed through DUK, a filtering program developed at JGI, which removes known Illumina sequencing and library preparation artifacts. The following steps were then performed for assembly: filtered Illumina reads were assembled using Velvet (version 1.2.07 [Zerbino and Birney, 2008]), 1–3 kb simulated paired end reads were created from Velvet contigs using wgsim (version 0.3.0 (https://github.com/lh3/wgsim)) and Illumina reads were assembled with simulated read pairs using Allpaths–LG (version r46652 [Gnerre and MacCallum, 2011]). The genomes of strains LMG 29879$^T$ and LMG 29880$^T$ were sequenced using the Illumina HiSeq 4000 platform. Sequence assembly of raw reads into contigs was performed with CLCgenomics workbench 7. The quality of the assemblies (draft genomes) and their DNA G+C content was assessed with Quality Assessment Tool for genome assemblies (QUAST (http://quast.bioinf.spbau.ru/)). The average nucleotide identity based on mummer (ANIm) values were calculated by JSpecies [Richter et al., 2015]. Genome annotation was performed by RAST [Aziz et al., 2008].

Phenotypic analysis

Biochemical characteristics were determined for one *Gilliamella* isolate of each MALDI–TOF MS cluster, i.e. LMG 28358$^T$, LMG 28359$^T$, LMG 29879$^T$ and LMG 29880$^T$, and for *G. apicola* NCIMB 14804$^T$. To test substrate utilization and enzyme activity, API 20NE galleries (bioMérieux) were inoculated according to the manufacturer's instructions. API 20NE strips were read after 2 days of incubation at 37°C. Gram–stain–reaction, verification of oxidase, catalase and DNase activity (DNase agar Difco™) and hydrolysis of Tween 20, Tween 40, Tween 60, Tween 80 (Tween 10 ml, peptone 10 g, NaCl 5 g, $CaCl_2.2H_2O$ 0.1 g and agar 9 g), starch (TSA agar supplemented with 10 g starch) and casein (PCA agar 23 g + dry skim milk 13 g) were performed using standard microbiological procedures [Macfaddinn, 1980]. Lactose fermentation was tested on MacConkey agar. Growth was checked on AC agar and M144 agar at different temperatures (20°C, 28°C, 37°C and 42°C). Growth was also tested on AC agar, M144 agar and Columbia blood agar at 28°C and 37°C in a micro–aerobic and anaerobic atmosphere. Cell morphology and motility was checked with phase contrast microscopy. To test the ability to degrade pectin or polygalacturonic acid, the strains were streaked onto MP–7 agar plates (HiMedia Laboratories) at pH 5 or 7 containing either pectin from citrus peel (Sigma) or polygalacturonic acid (Sigma). After two days of incubation, 1% hexadecyltrimethylammonium bromide (CTAB) was poured onto the plates. After 20 min, pectin or polygalacturonic acid degradation zones were measured with a mauser digital 2 micrometer. *Dickeya chrysanthemi* LMG 2804$^T$ was used as a positive control and as a negative control this strain was streaked on MP–7 agar plates without pectin. The ability to degrade polygalacturonic acid was also tested by growing the strains onto AC agar for two days after which an overlay agar layer containing polygalacturonic acid was poured onto the grown colonies as described by Engel et al. [2012].

Fatty acid methyl ester (FAME) analysis was performed for strains LMG 28358$^T$, LMG 28359$^T$, LMG 29879$^T$, LMG 29880$^T$ and NCIMB 14804$^T$ using an Agilent Technologies 6890N gas chromatograph. Cultivation of the strains and extraction of the fatty acids was performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI), except that fatty acids were extracted from cultures grown on M144 and AC agar at 37°C for 48h. The peaks of the profiles were identified using the TSBA50 identification library version 5.0 (MIDI).

The respiratory quinone composition was analysed for strains LMG 28358$^T$, LMG 28359$^T$, LMG 29879$^T$ and LMG 29880$^T$ using the protocol described by da Costa et al. [2011]. To improve the efficacy of the quinone extraction, the hexane and methanol phases were allowed to separate for two hours at –80°C. An XBridge BEH phenyl column (pore diameter 130Å, particle size 5 µm, 4.6 mm width x 250 mm length) was used to separate the respiratory quinones via high performance liquid chromatography (HPLC). The samples were eluted with 100% methanol at a rate of 1 min/ml and the quinones were detected at 269 nm. *G. apicola* NCIMB 14804$^T$ which was reported to comprise ubiquinone 8 was used as a control strain [Kwong and Moran, 2013].

### 4.5.4 Results and discussion

Spectra of isolates obtained from gut samples of wild bumblebees of *B. pascuorum*, *B. lapidarius* and *B. terrestris* caught near the city of Ghent, Belgium (Supplementary Table 4.3) grouped into four MALDI–TOF MS clusters (Figure 4.11 and Supplementary Table 4.3). Cluster two consisted of spectra of isolates from both a *B. lapidarius* and a *B. pascuorum* bumblebee while clusters one, three and four consisted of spectra of a single isolate each (obtained from a *B. lapidarius*, *B. terrestris* and a *B. pascuorum* bumblebee, respectively). Random amplified polymorphic DNA analysis revealed an identical DNA fingerprint for LMG 28359$^T$ and R-53705 which also grouped in the same MALDI-TOF MS cluster. Different DNA fingerprints were obtained for the other isolates, suggesting that each MALDI-TOF MS cluster represented a single strain (Supplementary Figure 4.20). For LMG 28359$^T$ and R-53705 this was confirmed by the identical 16S rRNA gene sequences. Comparative 16S rRNA sequence analysis also revealed that *G. apicola* NCIMB 14804$^T$ was their nearest phylogenetic neighbour. A phylogenetic tree based on the 16S rRNA gene sequences of strains LMG 28358$^T$, LMG 28359$^T$, LMG 29879$^T$ and LMG 29880$^T$ and their closest neighbours is shown in Figure 4.12. The latter phylogenetic tree also includes the 16S rRNA gene sequences of various novel *Gilliamella* strains isolated recently from north American bumblebee gut samples [Zheng et al., 2016]. The 16S rRNA gene sequence identities of strains LMG 28358$^T$, LMG 28359$^T$, LMG 29879$^T$, LMG 29880$^T$ and *G. apicola* NCIMB 14804$^T$ varied between 98.4 and 99.9%, demonstrating that the novel isolates should be classified into the genus *Gilliamella*.

To further elucidate the genotypic relationship between these five strains, draft genome sequences were determined and ANIm values were calculated (Table 4.9). The ANIm values of strains LMG 28358$^T$, LMG 28359$^T$, LMG 29879$^T$, LMG 29880$^T$ and NCIMB 14804$^T$ varied between 84.26 and 92.13%, indicating that each of these strains represents a distinct species [Richter and Rosselló-Móra, 2009; Richter et al., 2015]. The DNA G+C content of LMG 28358$^T$, LMG 29879$^T$, LMG 29880$^T$ and LMG 28359$^T$ is 34.6%, 34.6%, 35.5% and 35.9%, respectively, which is comparable to that of *G. apicola* NCIMB 14804$^T$ (33.6%).

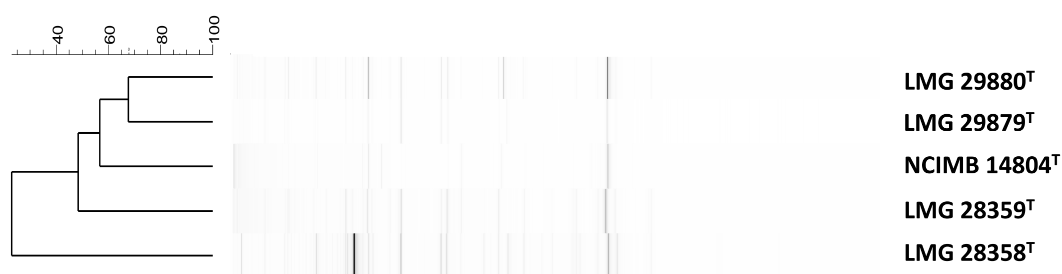



**Figure 4.11:** MALDI–TOF MS profiles of representative isolates of each MALDI–TOF MS cluster and *G. apicola* NCIMB 14804$^T$. Cluster analysis was performed by Pearson correlation and the hierarchical clustering method UPGMA.

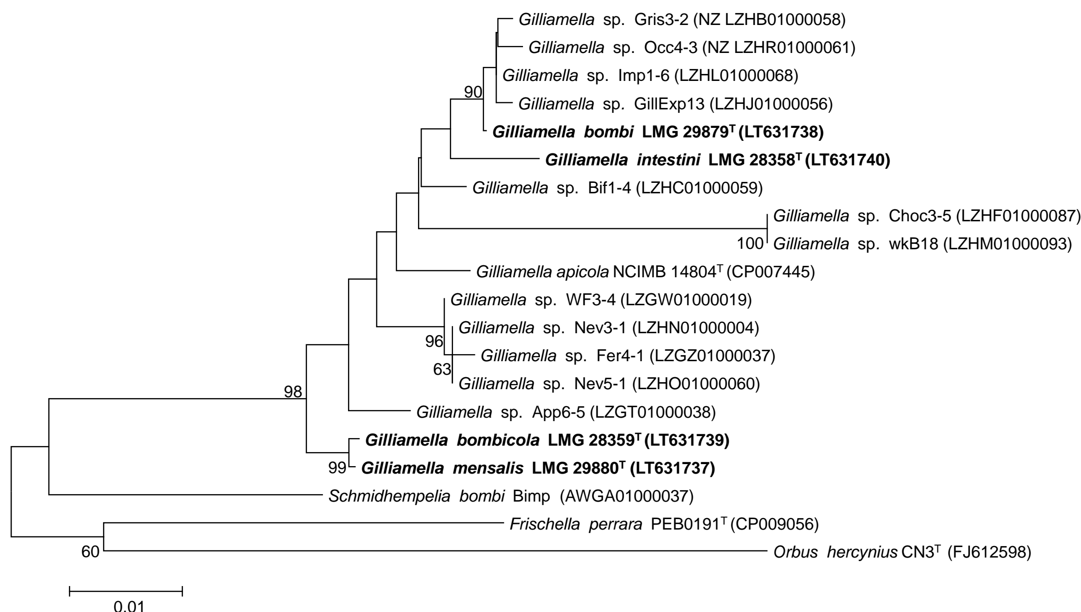



**Figure 4.12:** Phylogenetic tree based on 16S rRNA gene sequences of *G. intestini* LMG 28358$^{T}$, *G. bombicola* LMG 28359$^{T}$, *G. bombi* LMG 29879$^{T}$ and *G. mensalis* LMG 29880$^{T}$ and their closest neighbours. Included in the phylogenetic tree are also 16S rRNA gene sequences of *Gilliamella* strains isolated by Zheng et al. [2016]. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [Nei and Kumar, 2000]. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches if the percentage is 50% or higher. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor–Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.1000)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 78.6587% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1443 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [Tamura et al., 2013a].

**Table 4.9:** Similarity level of the 16S rRNA gene sequences and ANIm values of *G. intestini* LMG 28358$^{T}$, *G. bombicola* LMG 28359$^{T}$, *G. bombi* LMG 29879$^{T}$, *G. mensalis* LMG 29880$^{T}$ and *G. apicola* NCIMB 14804$^{T}$.

| | LMG 28358$^{T}$ | | LMG 28359$^{T}$ | | LMG 29879$^{T}$ | | LMG 29880$^{T}$ | | NCIMB 14804$^{T}$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 16S rRNA | ANIm | 16S rRNA | ANIm | 16S rRNA | ANIm | 16S rRNA | ANIm | 16S rRNA | ANIm |
| LMG 28358$^{T}$ | | | 98.6 | 87.53 | 99 | 87.79 | 98.5 | 92.12 | 98.5 | 85.54 |
| LMG 28359$^{T}$ | 98.6 | 87.93 | | | 98.6 | 91.57 | 99.9 | 87.55 | 98.4 | 85.73 |
| LMG 29879$^{T}$ | 99 | 87.83 | 98.6 | 91.59 | | | 98.5 | 87.07 | 98.7 | 84.27 |
| LMG 29880$^{T}$ | 98.5 | 92.13 | 99.9 | 87.56 | 98.5 | 87.06 | | | 98.4 | 84.26 |
| NCIMB 14804$^{T}$ | 98.5 | 85.77 | 98.4 | 85.56 | 98.7 | 84.27 | 98.4 | 84.26 | | |

As in *G. apicola* NCIMB 14804$^{T}$, the major fatty acids are $C_{16:0}$ and $C_{18:1}$ $\omega$7c (Supplementary Table 4.4). However, other fatty acids are also abundant for strain LMG 29880$^{T}$ ($C_{14\ :0}$ 3–OH and/or iso-$C_{16\ :1\ I}$) and strain LMG 28358$^{T}$ ($C_{12:0}$ and $C_{18\ :1}$ $\omega$9c). Although Kwong and Moran [2013] reported ubiquinone–8 as the predominant respiratory quinone of *G. apicola* NCIMB 14804$^{T}$, repeated analyses failed to yield quinones in any of the strains examined including *G. apicola* NCIMB 14804$^{T}$. Incomplete pathways for ubiquinone synthesis were detected in the genomes of the representative strains. The genes coding for the enzymes chorismate–pyruvate lyase (*ubiC*) and 2–octaprenyl–6–methoxyphenolhydroxylase (*ubiH*) were missing in each of these genome sequences including that of *G. apicola* NCIMB 14804$^{T}$. All five strains examined were Gram–stain negative, facultatively anaerobic, oxidase negative and non–motile rods. All strains were able to grow at 28°C and 37°C. None of the strains hydrolyzed Tween 20, 40, 60 or 80. Growth on media to test for DNAse activity and casein or starch hydrolysis was strain dependent; in case growth was observed there was no DNAse activity or casein or starch hydrolysis. As observed for *G. apicola* NCIMB 14804$^{T}$ [Kwong and Moran, 2013] our strains failed to grow in the API 20NE carbon substrate assimilation tests. However, this may be due to their inability to grow in the API AUX medium (bioMérieux). The phenotypic characteristics of the strains correspond with the ones reported earlier [Kwong and Moran, 2013]. Differential phenotypic characteristics between the representative strains and between these strains and *G. apicola* NCIMB 14804$^{T}$ are presented in Table 4.10.

**Table 4.10:** Differential phenotypic characteristics between *G. intestini* LMG 28358$^{T}$, *G. bombicola* LMG 28359$^{T}$, *G. bombi* LMG 29879$^{T}$, *G. mensalis* LMG 29880$^{T}$ and *G. apicola* NCIMB 14804$^{T}$.

| | LMG 28358$^{T}$ | LMG 28359$^{T}$ | LMG 29879$^{T}$ | LMG 29880$^{T}$ | NCIMB 14804$^{T}$ |
|---|---|---|---|---|---|
| Catalase | - | + | + | - | - |
| Esculin hydrolysis | - | - | - | - | + |
| $\beta$-galactosidase activity | + | - | - | - | + |
| Starch hydrolysis | No growth | No growth | - | - | No growth |
| Casein hydrolysis | No growth | - | - | - | No growth |
| DNase activity | No growth | No growth | - | - | No growth |
| Lactose fermentation | No growth | - | - | - | No growth |
| Growth at 20°C | + | - | w | w | + |
| Growth at 42°C | w | - | w | + | - |

+ = positive; – = negative; w = weak positive

The strains did not degrade pectin from citrus peel or polygalacturonic acid when grown on MP–7 medium or on AC agar with polygalacturonic acid. However, a complete pathway for pectate degradation was detected in the genome of LMG 28359$^{T}$ (Supplementary figure 4.21). In addition, the genome of LMG 29880$^{T}$ contains the *exuT* gene which codes for a hexuronate transporter which is responsible for transporting D–galacturonate into the cell and all genes for further degradation of D–galacturonate to pyruvate and glyceraldehyde–3–phosphate (Supplementary figure 4.21). In accordance with the analyses performed by Zheng et al. [2016], we screened the genomes of our strains for genes encoding enzymes necessary for the catabolism of xylose, arabinose, rhamnose and mannose. Neither of the four strains contained genes for xylose and rhamnose catabolism. In addition, incomplete pathways were detected for mannose catabolism in the genomes of strains LMG 29879$^{T}$, LMG 29880$^{T}$ and LMG 28358$^{T}$. While strain LMG 29879$^{T}$ only contains genes for enzyme IIA and IIB of the phosphotransferase system enzyme II, the genomes of strains LMG 29880$^{T}$, LMG 28358$^{T}$ and LMG 28359$^{T}$ also contain genes for the IIC and IID domain of the EII enzyme. The *manA* gene which codes for a mannose phosphate isomerase is only present in the genome of LMG 28359$^{T}$. Therefore, only strain LMG 28359$^{T}$ has the potential to catabolize mannose. The genes *araA*, *araB*, *araD* and *araT* which encode enzymes for the transport of arabinose into the cell and catabolism of arabinose to xylulose–5–phosphate were detected in the genome of LMG 29880$^{T}$.

Together, these data demonstrate that the isolates represent four novel species within the genus *Gilliamella* which can be distinguished by phenotypic, phylogenetic and genotypic characteristics. We therefore propose to classify the five isolates into the novel species *Gilliamella intestini*, with LMG 28358$^{T}$ as the type strain, *Gilliamella bombicola,* with LMG 28359$^{T}$ as the type strain, *Gilliamella bombi*, with LMG 29879$^{T}$ as the type strain and *Gilliamella mensalis*, with LMG 29880$^{T}$ as the type strain. Members of the genus *Gilliamella* have now been reported in honeybees and bumblebees and recently also in megachilid bees, flowers and during the process of wine fermentation [Godálová et al., 2016; McFrederick et al., 2016] suggesting that they can be horizontally transmitted between bees while visiting flowers. The data presented by Zheng et al. [2016] demonstrate that a considerable number of *Gilliamella* species awaits formal description (Figure 4.12).

### 4.5.5 Description of *Gilliamella intestini* sp. nov.

*Gilliamella intestini* (in.tes.ti'ni. L. gen. n. *intestini*, of the gut)

Growth on M144, AC and Columbia blood agar at 37°C in a micro–aerobic and anaerobic atmosphere after two days of incubation. Growth is also observed on these media at 20°C and 28°C although weaker. Weak growth is observed at 42°C after seven days of incubation. Strains are facultative anaerobic, Gram–stain negative, oxidase– and catalase negative and non–motile rods (0.5 µm wide and 1 µm long) and the colonies are shiny, white, round colonies (<1 mm). The type strain is negative for pectin and polygalacturonic acid degradation, Tween 20, 40, 60 and 80 hydrolysis, nitrate reduction, tryptophanase, glucose fermentation,

arginine dihydrolase, urease, esculin and gelatinase and did not grow on the media to test starch or casein hydrolysis, DNase activity and lactose fermentation. The type strain was positive for $\beta$–galactosidase. The DNA G+C content of the type strain is 34.6%. The accession numbers for the 16S rRNA gene sequence and the genome assembly of the type strain LMG 28358$^T$ (DSM 104029$^T$) are LT631740 and FMBA01000001–FMBA01000113, respectively. The Digital Protologue number of the type strain is TA00034.

### 4.5.6 Description of *Gilliamella bombicola* sp. nov.

*Gilliamella bombicola* (bom.bi'co.la. L. n. bombus a boom, a deep hollow noise, buzzing, also the zoological genus name of the bumblebee; L. suf. –cola [derived from incola, inhabitant] dwelling, occurring in; N.L. n. *bombicola* occurring in *Bombus*).

Strains are facultative anaerobes and are Gram–stain negative, non–motile, oxidase negative and catalase positive. The cells are rods (0.5 µm wide and 1 µm long) and the colonies are white, shiny, round and smaller than 1 mm after two days of incubation. Growth is observed on M144, AC and Columbia blood agar at 28°C and 37°C in a micro–aerobic and anaerobic atmosphere. Growth is not observed at 20°C and 42°C. The type strain is positive for esculin hydrolysis and is negative for pectin and polygalacturonic acid degradation, Tween 20, 40, 60 and 80 hydrolysis, nitrate reduction, tryptophanase, $\beta$–galactosidase, glucose and lactose fermentation, arginine dihydrolase, urease, casein hydrolysis and gelatinase and did not grow on the media to test starch hydrolysis and DNase activity. The DNA G+C content of the type strain is 35.9%. The accession numbers for the 16S rRNA gene sequence and the genome assembly of the type strain LMG 28359$^T$ (DSM 104085$^T$) are LT631739 and FMAQ01000001-FMAQ01000029, respectively. The Digital Protologue number of the type strain is TA00033.

### 4.5.7 Description of *Gilliamella bombi* sp. nov.

*Gilliamella bombi* (bom'bi. L. n. bombus a boom, a deep hollow noise, buzzing, also the zoological genus name of the bumblebee; N.L. gen. n. *bombi* of *Bombus*, of a bumblebee).

The colonies are white, round, 1 mm and shiny after two days of incubation and the cells are 0.5 µm wide and 1 µm long. Growth on M144, AC and Columbia blood agar at 28°C and 37°C in a micro–aerobic and anaerobic atmosphere after two days of incubation. Growth is weak at 20°C and 42°C and on AC agar in a micro–aerobic atmosphere at 28°C. Strains are facultative anaerobes and are Gram–stain negative, non–motile, oxidase negative and catalase positive. The type strain is negative for pectin and polygalacturonic acid degradation, Tween 20, 40, 60 and 80 hydrolysis and starch hydrolysis, nitrate reduction, DNase activity, tryptophanase, $\beta$–galactosidase, glucose and lactose fermentation, arginine dihydrolase, urease, casein hydrolysis and gelatinase. The DNA G+C content of the type strain is 34.6%. The accession numbers for the 16S rRNA gene sequence and the genome assembly of the type strain LMG 29879$^T$ (DSM 104030$^T$) are LT631738 and FMWS01000001–FMWS01000132, respectively. The Digital Protologue number of the type strain is TA00037.

### 4.5.8 Description of *Gilliamella mensalis* sp. nov.

*Gilliamella mensalis* (men.sa'lis. L. adj. *mensalis*, of a table; referring to the commensal relationship)

The cells are Gram–stain–negative, non–motile, catalase– and oxidase negative, rod–shaped and 0.5 µm wide and 1 µm long and the colonies are 1 mm, round, shiny and white after two days of incubation. Growth was observed after two days when incubated micro–aerobically and anaerobically on AC and Columbia blood agar at 28°C, 37°C and 42°C. Weak growth was observed at 20°C and on M144 agar. The type strain is negative for pectin and polygalacturonic acid degradation, Tween 20, 40, 60 and 80 and starch hydrolysis, nitrate reduction, DNase activity, tryptophanase, $\beta$–galactosidase, glucose and lactose fermentation, arginine dihydrolase, urease, casein hydrolysis and gelatinase. The DNA G+C content of the type strain is 35.5%. The accession numbers for the 16S rRNA gene sequence and the genome assembly of the type strain LMG 29880°(DSM 104442$^{T}$) are LT631737 and FMWR01000001–FMWR01000044 , respectively. The Digital Protologue number of the type strain is TA00036.

### 4.5.9 Acknowledgements

The authors acknowledge support by Ghent University-Special Research Fund (BOF).

### 4.5.10 Compliance with Ethical Standards

The present research involved sampling of bumblebees for which no permission was required as bumblebees are not included in the 'Decree of Species (het Soortenbesluit (http://codex.vlaanderen.be/Zoeken/Document.aspx?DID=1018227¶m=informatie])' of the Flemish government with inception on 01/09/2009. The authors do not have a conflict of interest.

The genome sequence data of LMG 28358$^{T}$ and LMG 28359$^{T}$ were produced by the US Department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) in collaboration with the user community. The work conducted by the U.S. Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

### 4.5.11 Supplementary material

**Supplementary Table 4.3:** Isolates, their isolation sources and MALDI–TOF MS cluster numbers.

| Isolates | Isolation source[a] | MALDI–TOF MS cluster numbers |
|---|---|---|
| LMG 28358$^T$ | Bumblebee H48 *B. lapidarius* | 1 |
| | Coupure Ghent (51.05130°NL/3.706°EL) | |
| LMG 28359$^T$ | Bumblebee H6 *B. pascuorum* | 2 |
| | Ledeganck botanical garden Ghent (51.0368°NL/3.7221°EL) | |
| R-53705 | Bumblebee H2 *B. lapidarius* | |
| | Ledeganck botanical garden Ghent (51.0368°NL/3.7221°EL) | |
| LMG 29879$^T$ | Bumblebee H14 *B. terrestris* | 3 |
| | Ledeganck botanical garden Ghent (51.0368°NL/3.7221°EL) | |
| LMG 29880$^T$ | Bumblebee H124 *B. pascuorum* | 4 |
| | Den Blakken Wetteren (51.0086°NL/3.899100°EL) | |

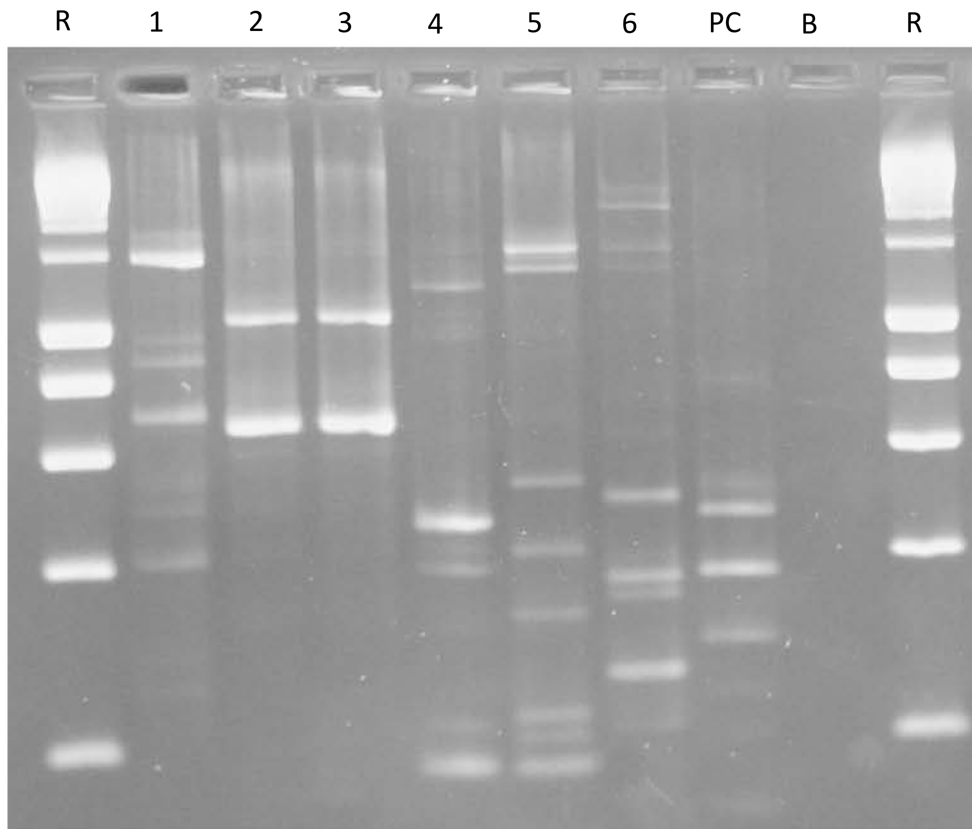
[a]Isolation source (bumblebee individual, bumblebee species and sampling location)

**Supplementary Table 4.4:** Fatty acids composition of *G. intestini* LMG 28358$^T$, *G. bombicola* LMG 28359$^T$, *G. bombi* LMG 29879$^T$ and *G. mensalis* LMG 29880$^T$, and of *G. apicola* NCIMB 14804$^T$.

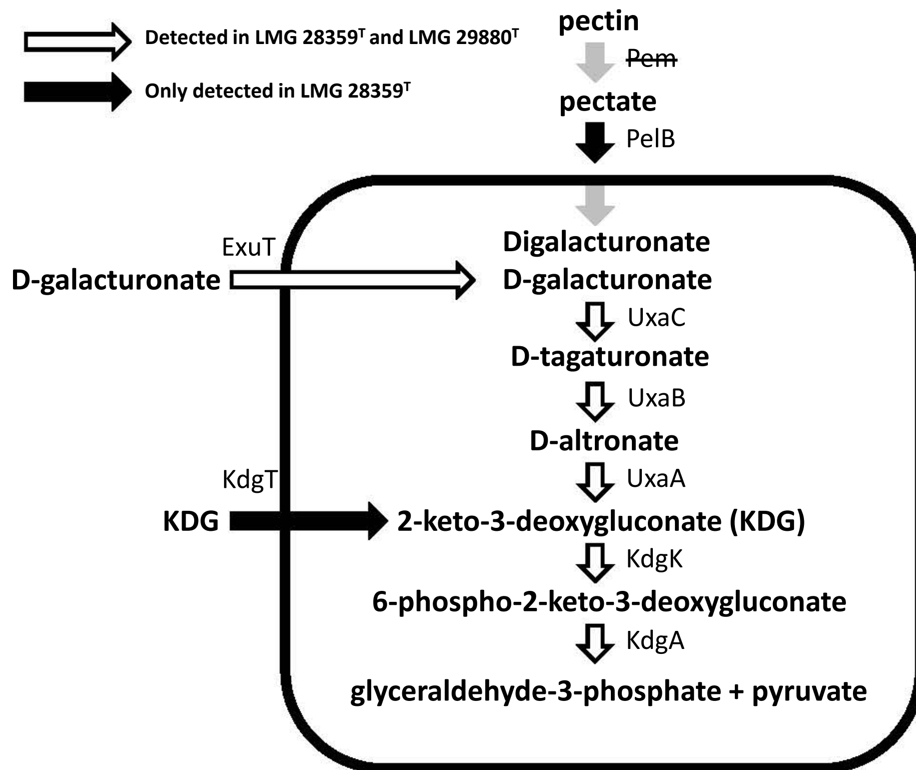
| | LMG 28358$^T$ | LMG 28359$^T$ | LMG 29879$^T$ | LMG 29880$^T$ | NCIMB 14804$^T$ |
|---|---|---|---|---|---|
| $C_{12:0}$ | 23.17 | 1.60 | 1.59 | 7.82 | ND |
| $C_{14:0}$ | 13.60 | 8.01 | 9.65 | 12.29 | 7.52 |
| $C_{16:0}$ | 27.60 | 35.62 | 35.19 | 29.63 | 31.69 |
| $C_{18:0}$ | ND | 1.81 | 1.33 | ND | 1.31 |
| $C_{18:1}$ $\omega$7c | 18.09 | 37.03 | 34.75 | 19.74 | 41.32 |
| $C_{18:1}$ $\omega$9c | 17.54 | 1.22 | 0.97 | ND | ND |
| $C_{14:0}$ 3–OH and/or iso–$C_{16:1\ I}$ | ND | 11.88 | 12.95 | 27.08 | ND |
| Iso–$C_{15:0}$ 2–OH and/or $C_{16:1}$ $\omega$7c | ND | 2.33 | 3.56 | 3.43 | 9.41 |
| $C_{18:0}$ ante/$C_{18:2}$ $\omega$6,9c | ND | 0.50 | ND | ND | ND |
| Other* | ND | ND | ND | ND | 8.75 |

*Includes $C_{12:0}$ aldehyde?/unknown 10.9525, iso–$C_{16:1\ I}$ and $C_{14:0}$ 3–OH

ND = not detected.




**Supplementary Figure 4.20:** RAPD profiles of the isolates belonging to the novel *Gilliamella* species. R = reference marker, 1 = *G. intestini* LMG 28358$^{T}$, 2 = *G. bombicola* LMG 28359$^{T}$, 3 = *G. bombicola* R-53705, 4 = *G. bombi* LMG 29879$^{T}$, 5 = *G. mensalis* LMG 29880$^{T}$, 6 = *G. apicola* NCIMB 14804$^{T}$, PC = positive control, B = blanc.




**Supplementary Figure 4.21:** Pectin degradation pathway. No pectin esterases (Pem) were detected to convert pectin to pectate. PelB = periplasmic pectate lyase, UxaC = urinate isomerase, UxaB = altronate oxidoreductase, UxaA = altronate hydrolase, KdgK = KDG kinase, KdgA = 2–dehydro–3–deoxyphosphogluconate aldolase, ExuT = hexuronate transporter, KdgT = KDG permease.

# Part IV

# General discussion and Future perspectives

# CHAPTER 5

## General discussion and Future perspectives

The yield of approximately two thirds of the world's agricultural crops relies on, or is improved by bee pollination [Goulson, 2003; Hatfield et al., 2012; Losey and Vaughan, 2006]. Intensive agriculture can lead to suboptimal crop production due to a shortage of wild bees and indirectly resulted in the domestication and commercialisation of several bee species [Lye et al., 2011]. Honeybees and bumblebees are the most widespread commercial pollinators. However, bumblebees are more efficient pollinators of certain crops and possess valuable characteristics for the pollination of greenhouse crops (Chapter 1). Because of the high density and potentially higher pathogen susceptibility of commercial bees, these bees can become reservoirs with high pathogen prevalence of which pathogens can spill back to wild bumblebees [Colla et al., 2006; Graystock et al., 2016b; Goulson and Hughes, 2015].

Several studies indicated that the bumblebee gut microbiota contribute to bumblebee health through pathogen inhibition, food digestion and detoxification of toxic sugars (Chapter 2) [Engel et al., 2012; Koch and Schmid-Hempel, 2011b; Zheng et al., 2016]. The implementation of probiotics may therefore reinforce the health of commercial bumblebees and increase their resistance against pathogens, which in turn may reduce pathogen spillback.

An efficient approach to obtain probiotic strains starts with the selection of a relevant isolation source and is followed by the isolation, identification and characterisation of its microbial community (Figure 5.1). *In vivo* tests are performed to confirm beneficial effects in the selected host and optimise administration conditions. The bee gut is the most evident isolation source to obtain probiotic strains for bees. If micro-organisms can be isolated from the bee gut, they can survive in this niche and potentially influence the gut environment. Other possible isolation sources are nectar and pollen as micro-organisms associated with bee food are naturally ingested by bees.

The aim of the present study was to properly inventorize the cultivable bumblebee gut microbiota, to identify them to the species-level and subsequently to start exploring their functional potential (Chapter 3). We also aimed to formally describe isolates which could

not be assigned to formally named species (Chapter 4). The availability of a collection of properly identified bumblebee gut isolates will facilitate the future exploration of the functional potential of these micro-organisms.

Section 5.1 discusses the isolation and identification of the bumblebee gut microbiota and presents a state of the art overview of its diversity. Section 5.2 reconsiders the observed functionality of the bumblebee gut microbiota and discusses its relevance. Finally, general conclusions and future perspectives are presented in section 5.3.

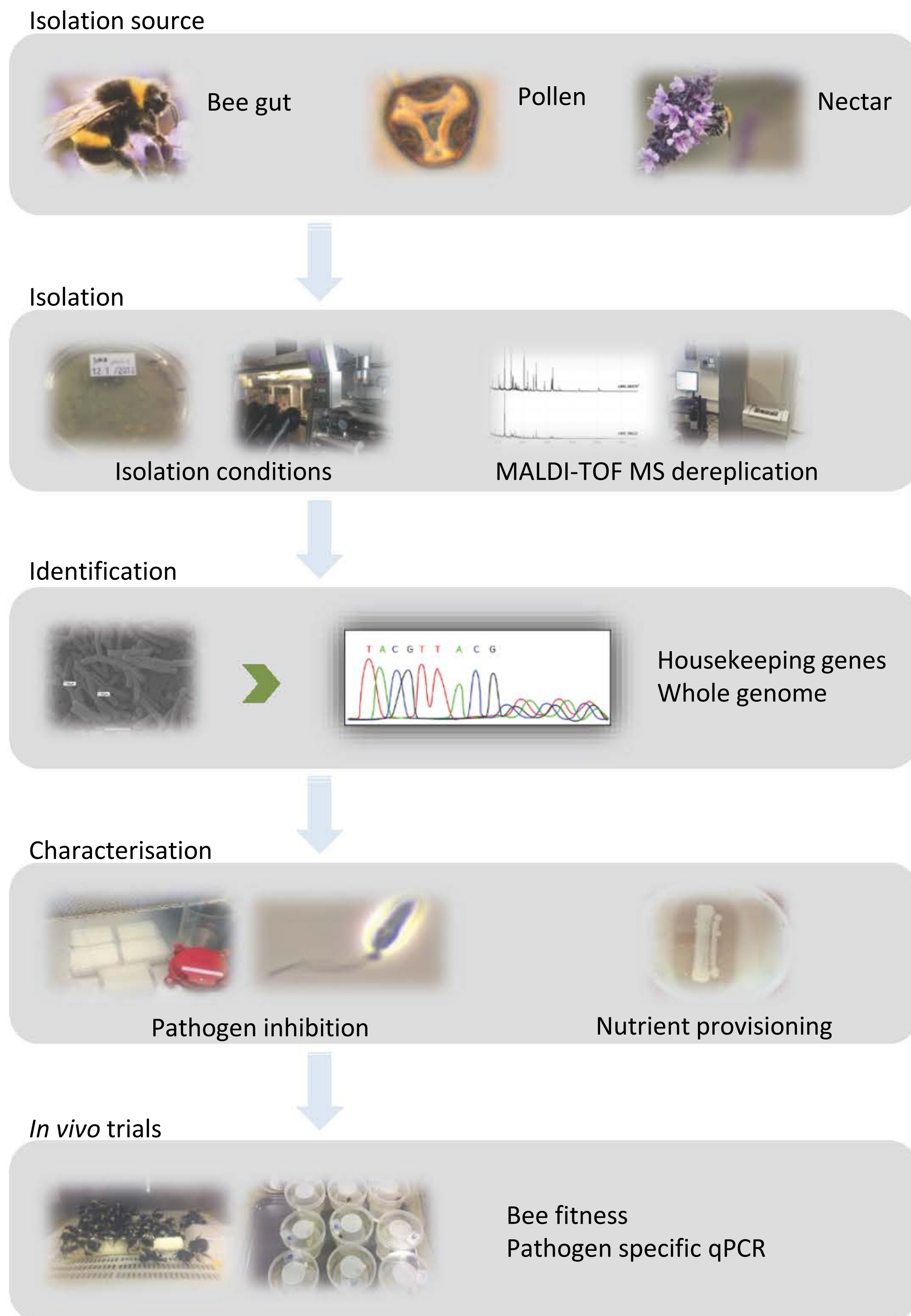



**Figure 5.1:** Schematic overview of an efficient approach to obtain bee probiotics.

## 5.1 Reflections on the isolation and identification of the cultivable bumblebee gut microbiota

### 5.1.1 Different factors influence the isolation success

Successful isolation of the cultivable gut microbiota of bumblebees depends on the diversity, treatment and number of samples collected, and on the selected isolation conditions. The sampling of different bumblebee species in different locations might enhance the successful isolation of probiotics because they might host a different microbial community and functionality. Subsequently, samples should be processed and preserved immediately and stored under proper conditions to prevent loss of microbial viability and diversity. Cell damage due to cryopreservation can be prevented by controlling the rate of cooling in freezing containers and adding cryo-protective agents like DMSO [Hoefman et al., 2013]. The number of samples and isolation conditions which are necessary to obtain an –ideally– complete image of the cultivable microbial diversity will depend on the species richness and evenness of the gut samples. Rarefaction curves can be determined alongside an isolation campaign to reveal if further sampling is still meaningful. Chapter 3 revealed that presumably 20 to 30 samples of every bumblebee species and location will be necessary to obtain a complete image of the cultivable gut microbiota of bumblebees.

At the start of this study, only few microbial species had been isolated from the bumblebee gut. These included *G. apicola*, *S. alvi*, *B. bombi*, *B. actinocoloniiforme*, *B. bohemicum* and *B. coagulans* and extensive isolation studies had mainly focused on *Bifidobacteriaceae* [Killer et al., 2009, 2010a, 2011; Kwong and Moran, 2013]. While Kwong and Moran [2013] used general media like blood agar and heart infusion agar, Killer et al. [2010b] used media selective for *Bifidobacteriaceae* like MTPY and a selective pollen medium. Different isolation media, ranging from the general TSB agar to LAB isolation media like tomato juice agar, all-purpose agar containing tween, and Rogosa agar were implemented by Olofsson et al. [2014] and Olofsson and Vásquez [2008] for the isolation of LAB from the honeybee crop. We evaluated several isolation conditions based on the isolation studies mentioned above, and information on bumblebee gut bacteria obtained by means of clone libraries and 16S rRNA amplicon sequencing studies. The latter studies indicated that apart from some Alpha-, Beta- and Gammaproteobacteria, most of the bumblebee gut bacteria were LAB [Koch and Schmid-Hempel, 2011b; Kwong and Moran, 2016a; Martinson et al., 2011; Olofsson and Vásquez, 2008].

In a preliminary study (data not shown), we used two general media (AC aerobic and microaerobic and BHI agar microaerobic) together with media for the selective isolation of LAB (MRS + 0.1% sorbic acid aerobic and anaerobic), fructophilic LAB (MRS + 2% fructose aerobic and anaerobic) and *Bifidobacteriaceae* (M144 and MTPY agar anaerobic). The transfer of micro-organisms from the bumblebee gut to a solid medium in the lab can be stressful and might be facilitated for some bacteria by pre-incubation in liquid medium.

In addition, some isolation studies observed more colony forming units and detected novel bacteria when using gellan gum instead of agar [Tamaki et al., 2005]. We therefore also evaluated pre-incubation in liquid medium and the use of gellan gum instead of agar as a gelling agent as potential isolation conditions. However, we did not isolate additional species when applying these alternative isolation conditions. Furthermore, as the obtained diversity on AC agar and MTPY agar was higher than that on BHI agar and on M144, respectively, the latter two media were not further applied.

It is worth noting that of the 3612 isolates picked up from the primary isolation plates, only 64% (2301 isolates) grew after subcultivation, and could be subjected to MALDI-TOF MS dereplication. When a significant number of picked isolates failed to grow upon transfer, isolates which were picked again from the same primary isolation plate were often identified as *L. bombicola*, *B. coagulans* and *Gilliamella* spp. This might indicate that a significant loss of isolates was mainly caused by poor growth of these species. In addition, the same organisms also regularly failed to grow after preservation in Microbank$^{TM}$ cryogenic vials. Further analyses demonstrated (data not shown) that recovery and growth of *Gilliamella* isolates was enhanced when the medium was buffered with 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and activated charcoal was added to the medium. This indicates that the production of acids or toxic compounds might impede good growth of these species.

### 5.1.2 MALDI-TOF MS as a high-throughput dereplication tool

To speed up the identification process and its throughput capacity, and to reduce costs and labour, it is recommended to first dereplicate isolates (i.e. the rapid grouping of indistinguishable isolates) in large-scale isolation campaigns. MALDI-TOF MS dereplication (Figure 5.2) allows the grouping of isolates at the species-level based on ionized low-molecular-weight protein profiles [Ghyselinck et al., 2011]. Protein extracts are made for each isolate, are spotted twice onto a MALDI-TOF MS plate consisting of 384 positions and are overlaid with matrix solution. The preparation and spotting of two-hundred protein extracts and subsequent measurement of the plate can easily be performed in one day. This is a major advantage compared to other dereplication tools like REP-PCR which requires high quality DNA extracts, is limited to approximately 14 samples per gel, is labour-intensive and may therefore take several days. The resolution of MALDI-TOF MS is situated at that of the species to strain level [Ghyselinck et al., 2011]. Although variations in the protein profiles due to differences in culture age or growth medium can occur, this does not affect species-level differentiation [Wieme et al., 2014]. Strain-level differentiation, however, is complicated by experimental variations which affect the presence and intensity of strain-specific peak classes and also depends on the investigated species [Ghyselinck et al., 2011; Wieme et al., 2014]. In the present study, the AB Sciex 4800 MALDI TOF/TOF$^{TM}$ Analyzer was used which was mainly developed for high-throughput proteomic analyses. The system therefore does not include an identification database or tools to analyse the mass spectra. Good quality MALDI-TOF MS profiles were obtained for 84% of the isolates and were imported in the Bionumerics v5.1 software package, and curve-based clustering was performed. Repeated

MALDI-TOF MS analyses of a selection of isolates of which no good profiles were obtained, revealed the difficult protein-extraction of *L. bombicola* isolates.

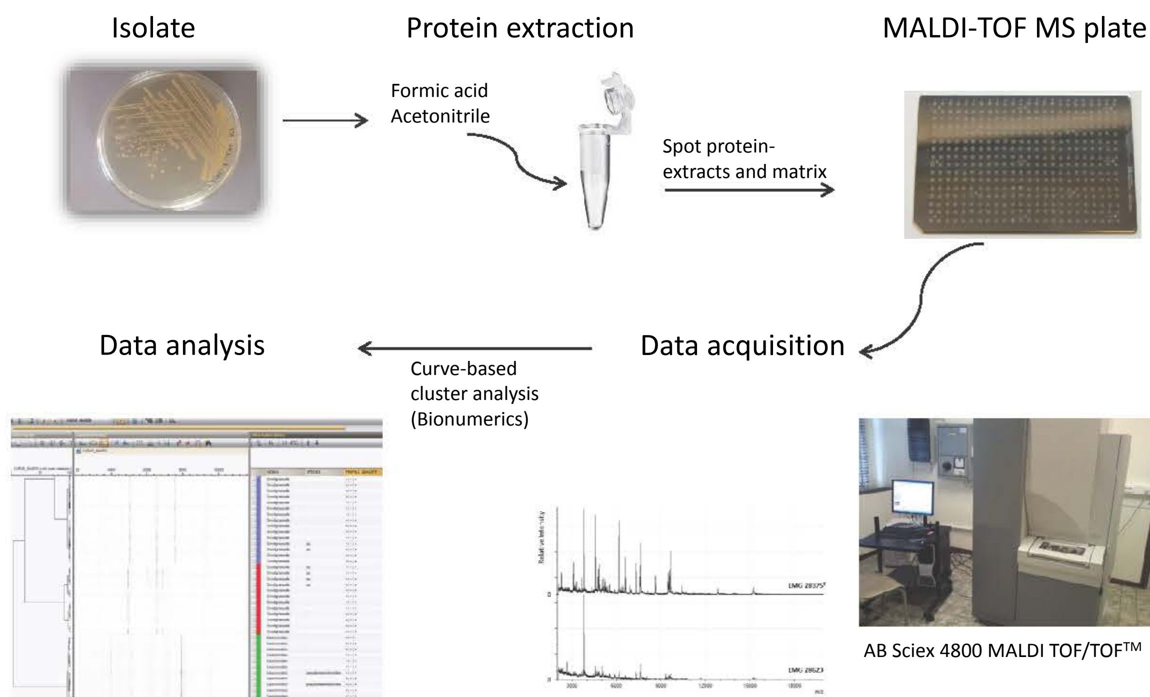



**Figure 5.2:** MALDI-TOF MS dereplication.

### 5.1.3 Towards an accurate identification

The accurate identification of potentially probiotic isolates is important to make a proper selection of isolates for subsequent functional analyses and to explicitly assign functional characteristics to specific isolates of formally named species. The 16S rRNA gene is the most common gene used for bacterial identification because it is universally present, is conserved due to its essential function and is not prone to recombination. However, this gene has a low resolution at the species level and different species can have highly similar 16S rRNA gene sequences [Clarridge, 2004]. In the present study, a full 16S rRNA gene sequence was determined for each species isolated and was deposited in a public database. Because most of the obtained species were LAB and bifidobacteria, a more accurate identification was performed for these bacteria by sequencing a 455 bp fragment of the *pheS* gene for members of the genera *Leuconostoc*, *Fructobacillus*, *Lactococcus*, *Enterococcus*, *Vagococcus* and *Lactobacillus* and a 445 bp fragment of the hsp60 gene for members of the *Bifidobacteriaceae*. The *pheS* and *hsp60* genes are housekeeping genes with a higher taxonomic resolution than the 16S rRNA gene and have been proven useful to differentiate species of these genera

[Naser et al., 2007; Zhu, 2003]. Housekeeping genes like the *pheS* and *hsp60* gene encode for proteins with essential functions and are conserved among bacteria. The development of universal primer sets for these genes is, however, not always possible which together with their taxon-specific resolution limits their use to specific taxa. Primer-related issues are overcome by sequencing whole genomes which is increasingly becoming cheaper, and might become a routine practice to accurately identify bacterial isolates in the near future. The availability of whole genome sequences facilitates the use of robust genome-based measures for relatedness, like multilocus sequence and average nucleotide identity analyses (Chapter 4).

Next to DNA-based approaches, proteomics tools like MALDI-TOF MS may allow a rapid and reliable identification. In the present study, representative isolates from each MALDI-TOF MS cluster were identified by 16S rRNA gene sequence analysis which precluded the need to sequence the 16S rRNA gene of all isolates, a commonly applied but relatively expensive identification procedure in many studies [Corby-Harris et al., 2014; Zheng et al., 2016]. The obtained identification results were incrementally introduced in the growing MALDI-TOF MS database which facilitated direct identification of isolates belonging to these species in subsequent analyses. Analysis of the MALDI-TOF MS spectra allowed to discriminate between the isolates at the species level and for some species like *Snodgrassella alvi* even at the infraspecific level. Also the four *Gilliamella* species which share a high degree of 16S rRNA gene sequence similarity were reliably differentiated by MALDI-TOF MS.

To identify the obtained yeast species, a 600 bp D1/D2 region of the 26S rRNA gene was sequenced and analysed using the Fungalbarcoding database. *Ascomycetous* yeasts (e.g. *Candida* and *Zygosaccharomyces*) are considered identified if their partial 26S rRNA gene sequences are more than 99% similar to that of the type strain [Kurtzman and Robnett, 1998; Lopandic et al., 2006]. In the present study, the ITS1 region, which is more variable and confers a higher resolution than the 26S rRNA gene, was sequenced (data not shown) and confirmed the identification of *C. bombi* and *Z. rouxii* isolates. A resolution similar to that of the 26S rRNA gene was observed as the ITS1 sequences were also more than 99% similar to that of the respective type strains. No ITS1 sequence was publicly available for *C. bombiphila* and many fungal species remain unrepresented in fungal databases which limits the current use of molecular barcodes for identification [Nilsson et al., 2006; Hawksworth, 2015]. Yeast isolates belonging to two distinct MALDI-TOF MS clusters could not be identified and were referred to as *Candida* sp. I (94% 26S rRNA gene sequence similarity to *C. bombiphila*) and II (95.6% 26S rRNA gene sequence similarity to *C. etchelsii*). They likely represent novel species based on their low 26S rRNA gene sequence similarity to currently described species. Most fungal taxonomic studies have been based on morphology and although it is currently highly recommended to make cultures and molecular data publicly available, there are no such formal requirements for the description of novel yeast species [Seifert and Rossman, 2010].

### 5.1.4 From a polyphasic to a genomic taxonomy

In the course of our isolation campaign, bacterial isolates which could not be assigned to an established species were formally described and named by using the required polyphasic taxonomic studies (Chapter 4). Taxonomy includes the identification, classification and naming of organisms [Vandamme et al., 1996]. Its ultimate goal is to group organisms which are phylogenetically related and phenotypically and genotypically similar. This allows an efficient communication about micro-organisms and an association of ecological characteristics to specific taxa. Bacterial species are difficult to define and species concepts are primarily practical tools which should allow a consensus approach for classification. Polyphasic taxonomy is such an approach in which a bacterial species is defined as a monophyletic group of strains which share more than 98.65% 16S rRNA gene sequence similarity and which are genetically (>96% average nucleotide identity) and phenotypically similar [Kim et al., 2014; Vandamme and Peeters, 2014].

Measures of whole genome similarity are required if the 16S rRNA gene sequence similarity is above or close to the 98.65% threshold. DNA-DNA hybridization (DDH) as applied in Section 4.2 to determine genomic similarity requires large amounts of DNA and is time-consuming, labor-intensive and limited to specialized laboratories. With the availability of bacterial genomes, this approach has been replaced by overall genome relatedness indices (OGRI) like ANIm (as applied in Section 4.5). ANI is a robust measure for species delineation because it strongly correlates with 16S rRNA gene sequence similarity and the mutation rate of the genome. ANI calculation does not require complete whole genome sequences (at least 50% genome coverage for both strains is recommended) and the threshold of 95-96% ANI corresponds to a DDH value of 70% [Richter et al., 2015; Richter and Rosselló-Móra, 2009]. Two methods are routinely available to calculate ANI values, ANIblast (ANIb) and ANImummer (ANIm). Although similar values are obtained for both methods, ANIm is preferable in the ANI ranges >90% [Richter et al., 2015]. ANIm calculates an average genomic similarity measure based on the similarity of maximal matches between two genomes while ANIb artificially cuts the genome into 1020 nucleotide fragments before the similarity of these fragments is calculated based on BLAST analyses [Goris et al., 2007; Richter et al., 2015; Richter and Rosselló-Móra, 2009]. While the latter approach was developed to mimic the wet-lab DDH where genomic DNA is sheared in fragments of about 1020 nucleotides, it is unclear why some authors continue to consider it meaningful to address whole genome sequence similarity this way. Another often used OGRI is genome blast distance phylogeny (GBDP) [Meier-Kolthoff et al., 2013]. GBDP calculates an overall distance measure by transforming the distances calculated for high-scoring segment pairs (HSPs). These HSPs are intergenomic matches and result from local alignment based on BLAST. An advantage of GBDP over ANI is that it is less likely to be affected by horizontal gene transfer by the implementation of a correction for paralogy. In case of an overlapping hit with an orthologues and with a paralogues gene, only the best hit (which is most likely the orthologues gene) is selected [Meier-Kolthoff et al., 2013]. The availability of genome sequences allows the

fast determination of genomic similarity precluding the need to perform DDH. Also the G+C content of a genome, which is a compulsory value for species descriptions, is easily retrieved from the genome sequence. Species descriptions often require specific phenotypic tests for particular taxa like fructose-6-phosphate phosphoketolase activity determination for bifidobacteria (applied in section 4.3), analysis of SCFA production (applied in section 4.2 and 4.3), quinone and FAME (applied in section 4.1, 4.4 and 4.5). These are time- and money-consuming tests which are often poorly discriminative between species and merely serve a descriptive purpose.

A species description based on a genomic taxonomy approach should therefore be limited to the determination of a robust OGRI and a minimal phenotypic description which includes cultivation conditions and relevant functional traits like virulence. In addition, the genome sequence and type strain should be deposited into public databases and culture collections, respectively. Recently, the Digital Protologue Database (DPD) has been developed and allows the deposition of protologues of described species and candidate species [Rosselló-Móra et al., 2017]. This constantly evolving and curated database will enhance the retrieval of taxonomic information and the comparison of prokaryotic characteristics.

### 5.1.5 Evaluation of the isolation approach through 16S rRNA amplicon sequencing

To evaluate the success of the isolation campaign, 16S rRNA amplicon sequencing was performed on samples pooled according to bumblebee species. Reads of approximately 460 bp were obtained by Illumina MiSeq paired-end sequencing of 2 x 300 bp of the V3-V4 region and grouped into 2137 OTUs. OTUs with less than 0.5% of the reads were not considered further which resulted in 28 OTUs that were retained. The 0.5% cut-off was based on analysis of a mock community in which OTUs with less than 0.5% of the reads were mainly singletons and doubletons. Although some genuine low abundant OTUs might also be removed by this approach, mainly OTUs based on sequencing errors will be eliminated this way.

Some micro-organisms were only detected through isolation or through amplicon sequencing. Also in this era of metagenomics, a combination of cultivation dependent and independent methods therefore remains optimal to obtain a more complete image of the microbial diversity [Hugon et al., 2013; Lagier et al., 2015]. Isolates were obtained for most of the detected, and previously reported, phylotypes which underscores the validity of the isolation conditions for most of the bacteria present [Meeus et al., 2015; Martinson et al., 2011; Moran et al., 2012; Koch and Schmid-Hempel, 2011b]. Most of the phylotypes for which no representatives were isolated represent a minority of the sequencing reads, are rarely reported in the bumblebee gut, and therefore presumably correspond to environmental species. Two of these low abundant phylotypes (Gamma-II and Lacto-4) are however commonly detected in the bumblebee gut [Koch and Schmid-Hempel, 2011b; Meeus et al., 2015] but no representative isolates are available yet. The isolation conditions used are either not suitable for these bacteria or these

bacteria were overgrown by other, more dominant bacteria. The 16S rRNA gene sequence of the Gamma-II phylotype is approximately 91% similar to that of species within the genera *Pseudomonas* and *Azotobacter* [Koch and Schmid-Hempel, 2011b]. Although species within these genera are aerobic or facultative anaerobic, it is possible that the Gamma-II phylotype consists of a novel obligate anaerobic genus preventing the isolation of representatives in the present study. Also the Lacto-4 phylotype may correspond to an obligate anaerobic genus. A dissection of the guts of three bumblebees and isolation on MRS and MTPY agar was performed under strict anaerobic atmosphere in an anaerobic workstation but only isolates of *L. bombicola*, *B. coagulans* and *Gilliamella* spp. were obtained.

It has been claimed for a long time that only a minority of the bacteria found in nature can be cultivated in the lab; the latter problem has been referred to as the 'great plate count anomaly'[Staley and Konopka, 1985]. Recent advances in cultivation approaches try to tackle this by using isolation conditions that mimic the physiological conditions of the isolation source, by using a highly diverse and large set of isolation conditions or by co-cultivation experiments with other bacteria [Stewart, 2012; Lagier et al., 2015, 2016]. The bumblebee gut micro-organisms are isolated from a complex environment where they interact with the bumblebee host and its intestinal microbial community. The failure to cultivate some bacteria may therefore be explained by unsuitable isolation conditions used in the present study or the absence of essential compounds which are present in the bumblebee gut.

In addition to the pooling of the samples according to bumblebee species, the pooling of the crop and gut of each bumblebee may partially explain the isolation of bacteria which were not detected by 16S rRNA amplicon sequencing. As especially the hindgut of bees contains a high load of micro-organisms [Martinson et al., 2012], the smaller number of micro-organisms present in the crop could have been masked in the 16S rRNA amplicon sequencing analysis [Manter et al., 2010]. These less abundant micro-organisms might be isolated if the provided isolation conditions select for them.

### 5.1.6 Diversity of the bumblebee gut microbiota: todays' state of the art

Nowadays, the interest in the microbial diversity of many environments is increasing due to the advances in 16S rRNA amplicon sequencing approaches. Influenced by the length and region of the sequenced 16S rRNA gene fragment and the cut-off used to obtain OTUs, the taxonomic resolution of 16S rRNA amplicon sequencing is limited to that of the genus or even family level [Clarridge, 2004; Mysara et al., 2016]. Amplicon sequencing of the full 16S rRNA gene sequence allows to improve the resolution of the data and is already possible by PacBio SMRT DNA sequencing [Schloss et al., 2015]. Although the per base sequencing cost is still significantly higher than for Illumina MiSeq, it is likely that the PacBio SMRT DNA sequencing will be increasingly used in future studies [Schloss et al., 2015]. As described in the introduction (part 1), especially 16S rRNA amplicon sequencing studies demonstrated that the gut microbiota composition of honeybees and bumblebees is similar and consists of core and non-core phylotypes. In the course of the present PhD study,

cultivated representatives have become available for several of these phylotypes which often represent multiple species (Table 5.1). Although amplicon sequencing is extremely popular, it is generating high numbers of sequences which cannot be assigned to validly named species and cultivation remains necessary to reveal the role of these micro-organisms [Lagier et al., 2016].

We performed a thorough isolation campaign of the gut microbiota of 60 bumblebees comprising four *Bombus* species using the above mentioned isolation conditions and obtained and examined 1940 isolates which represented 52 species (Chapter 3). These species constituted a diverse group of micro-organisms belonging to the Alpha-, Beta- and Gammaproteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Table 5.1). The most commonly isolated bacterial species were *L. bombicola*, *F. fructosus*, *B. coagulans*, *S. alvi*, *F. tropeaoli*, *Gilliamella* spp. and *L. kunkeei*. Multiple MALDI-TOF MS clusters were obtained for several of these species, indicating multiple strains were isolated.

In addition to the 60 bumblebees identified as *B. terrestris*, *B. lucorum*, *B. lapidarius* and *B. pascuorum*, we also analysed the microbiota of four *B. hypnorum* bumblebees (data not shown). The 95 isolates however mainly belonged to the most commonly isolated species. As no novel species were obtained from these samples and since we could collect only four *B. hypnorum* bumblebees, these data were not included in Chapter 3.

Table 5.1 presents a state of the art overview of phylotypes detected in the bumblebee and honeybee gut and cultivated species corresponding to these phylotypes. It clearly indicates that in the present PhD study cultivated representatives were obtained for most of the reported phylotypes and a more accurate identification of the bumblebee gut microbionts has been provided. Several phylotypes like Firm-S and Firm-E of which the 16S rRNA gene sequences matched with multiple species each, are now represented by cultivated representatives identified to the species level [Meeus et al., 2015].

We isolated several species which were not reported earlier in the bumblebee gut and which might be environmental rather than true endosymbionts. Several of these have indeed been obtained from environmental sources like flowers, soil and water (Table 5.2) and most of them are LAB (n = 21). *F. fructosus*, *F. tropaeoli*, *L. lactis* and *L. kunkeei* were isolated from several bumblebees in the course of this PhD study. Although *F. fructosus* and *L. kunkeei* are abundant members of the honeybee crop and *L. lactis* has been observed in the bumblebee gut, to our knowledge *F. tropeaoli* has never been detected in honeybees or bumblebees [Endo and Salminen, 2013; Meeus et al., 2015]. Presumably these LAB are present in the bumblebee crop and are therefore not or only sporadically detected by 16S rRNA amplicon sequencing in which especially hindgut bacteria will be detected due to their higher abundance [Martinson et al., 2012; Manter et al., 2010].

We unintentionally also isolated yeasts belonging to the genera *Candida* and *Zygosaccharomyces*. Our data suggest that specific yeast species can be highly prevalent in the bumblebee gut. Brysch-Herzberg [2004] showed that ascomycetous yeasts belonging to the

genera *Metschnikowia*, *Candida* and *Zygosaccharomyces* were associated with the plant-bee environment. While *Zygosaccharomyces rouxii* also occurs in soil, *Metschnikowia kunwiensis* and *Candida bombi* are closely associated with bumblebees and depend on bumblebees to survive the winter [Brysch-Herzberg, 2004].

**Table 5.1:** Bacterial phylotypes in the gut of bumblebees and honeybees and the cultivated or candidate species assigned to these phylotypes.
Species isolated in the present thesis are indicated in bold. The names of the phylotypes are those used by Meeus et al. [2015] unless stated otherwise. If the phylotype belongs to the core gut microbiota, the host name is indicated in bold character type.

| Phylotype (closest relative) | Cultivated or named species | Host |
|---|---|---|
| Gamma-1 | | **H,B** |
| | *Gilliamella apicola* [Kwong and Moran, 2013] | H,B |
| | ***G. mensalis*, *G. bombicola*** | B |
| | ***G. bombi*, *G. intestini*** | B |
| | Candidatus *Schmidhempelia bombi* [Martinson et al., 2014] | B |
| Gamma-2 | *Frischella perrara* [Engel et al., 2013] | H |
| Gamma-II[a] | | |
| (*Pseudomonas* sp. 91%)[b] | / | H,B |
| Gamma-P | | |
| (*Pseudomonas* spp. 100%)[c] | / | B |
| Gamma-E1 | | |
| (*Buttiauxella agrestis* 100%)[c] | / | B |
| Gamma-E2 | ***Hafnia alvei*** | H,B |
| Gamma[d] | ***Serratia marcescens*** | B |
| Beta | ***Snodgrassella alvi*** [Kwong and Moran, 2013] | **H,B** |
| Alpha-1 | *Bartonella apis* [Kesnerova et al., 2016] | H |
| Alpha-2.1 | | |
| (*Gluconobacter* sp.)[e] | / | H |
| Alpha-2.2 | | H,B |
| | *Parasaccharibacter apium* [Corby-Harris et al., 2014] | H |
| | ***Bombella intestini**** | B |
| Firm-5/Lacto-1 | | **H,B** |
| | *Lactobacillus kullabergensis*, *L.melliventris*, | H |
| | *L. kimbladii*, *L. helsingborgensis* [Olofsson et al., 2014] | H |
| | ***L. apis*** | H |
| | ***Lactobacillus bombicola*** | B |
| Firm-4/Lacto-2 | | **H**,B |
| | ***Lactobacillus bombi*** [Killer et al., 2014] | B |
| | *L. mellis* [Olofsson et al., 2014] | H |
| Bin4[f] | *Lactobacillus mellifer* [Olofsson et al., 2014] | H |
| Lacto-3 | | H,B |
| | ***Lactobacillus kunkeei*** [Endo and Salminen, 2013], | H |
| | *L. apinorum* [Olofsson et al., 2014] | H |
| Lacto-4 | | |
| (*Lactobacillus* sp. 89%)[b] | / | H,B |
| Lacto-5 | ***Lactobacillus kimchicus*** | H,B |

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| Phylotype (closest relative) | Cultivated or named species | Host |
|---|---|---|
| Fructobacillus[b] | ***Fructobacillus fructosus*** [Endo and Salminen, 2013] | H,B |
| Firm-S | ***Lactococcus lactis*** | B |
| Firm-E | ***Enterococcus faecalis*** | B |
| Bifido-1 | | **H**,B |
| | ***Bifidobacterium actinocoloniiforme*** [Killer et al., 2011] | B |
| | *B. asteroides*, *B. coryneforme*, *B. indicum* [Vasquez et al., 2012] | H |
| Bifido-2 | | H,B |
| | ***Bifidobacterium bombi*** [Killer et al., 2009] | B |
| Bifido-3 | ***Bombiscardovia coagulans*** [Killer et al., 2010a] | **B** |
| Bifido-4 | | H,B |
| | *Bifidobacterium bohemicum* [Killer et al., 2011] | B |
| Bifido-X | ***Bifidobacterium commune*** | H,B |
| Bacteroidetes | | H,B |
| | *Apibacter adventoris* [Kwong and Moran, 2016b] | H |
| | ***A. mensalis*** | B |

H = honeybee, B = bumblebee.

[a] Gamma-2 in Koch and Schmid-Hempel [2011b].

[b] Identity based on a full 16S rRNA gene sequence [Koch and Schmid-Hempel, 2011b].

[c] Identity based on a partial 16S rRNA gene sequence (253 bp) [Meeus et al., 2015].

[d] Phylotype detected by Li et al. [2015].

[e] No sequence available in public databases.

[f] Phylotype detected by Olofsson and Vásquez [2008].

[*] *B. intestini* is the only validated genus and species corresponding to the Alpha-2.2 phylotype.

**Table 5.2:** Additional microbial species isolated in this thesis.

| Phylum | Species | Previously detected niches |
|---|---|---|
| Fungi | *Candida bombi* | bumblebees, flowers |
| | *Candida* sp. | / |
| | *Candida bombiphila* | bumblebees, flowers |
| | *Zygosaccharomyces rouxi* | bumblebees, flowers, food, beverages |
| Firmicutes | *Fructobacillus tropaeoli* | flowers |
| | *Leuconostoc mesenteroides* | dairy products, plants |
| | *Leuconostoc pseudomesenteroides* | food |
| | *Leuconostoc* sp. | / |
| | *Weissella bombi* | / |
| | *Weissella viridescens* | meat |
| | *Convivina intestini* | / |
| | *Lactococcus garviae* | tonsils and faeces of animals, food |
| | *Vagococcus entomophilus* | wasp digestive tract |
| | *Bacillus licheniformis* | soil, food, water, cow and rodent feces |
| | *Bacillus niacini* | soil |
| | *Bacillus smithii* | soil, food, human intestine |
| | *Bacillus* spp. | / |
| | *Paenibacillus barengoltzii* | spacecraft assembly facility |
| | *Lysinibacillus fusiformis* | soil |
| | *Ureibacillus suwonensis* | compost |
| Alphaproteobacteriaceae | *Commensalibacter intestini* | intestine of *D. melanogaster* |
| Gammaproteobacteriaceae | *Rosenbergiella nectarea* | floral nectar |
| | *Enterobacter kobei* | clinical specimens |
| | *Escherichia vulneris* | wounds of humans and animals |
| | *Moraxella osloensis* | symbiont of the slug–parasitic nematode *Phasmarhabditis hermaphrodita* |
| | *Morganella morganii* | environment, intestine of humans, animals and reptiles |
| Actinobacteria | *Micrococcus luteus* | water, soil, air |
| | *Kocuria rhizophila* | rhizosphere of narrowleaf cattail (*Typha angustifolia*) |
| | *Streptomyces albidoflavus* | soil |
| | *Streptomyces thermodiastaticus* | soil |

## 5.2 Functionality of the bumblebee gut microbiota

*Li et al.* reported the occurrence of two bumblebee gut enterotypes of which one was dominated by *Gilliamella* and *Snodgrassella* and the other by *Enterobacteriaceae*. Although the presence of these enterotypes was not apparent in our dataset, the prevalence of species belonging to the *Enterobacteriaceae* was higher in *B. lucorum* and *B. terrestris* samples while in the *B. terrestris* sample more than 70% of the reads consisted of a *Hafnia* and *Enterobacter* phylotype. Based on NMDS and ANOSIM analyses of presence/absence data, we observed a significant clustering of cultivable microbial gut communities according to bumblebee species. A different gut microbiota composition and richness between certain bumblebee species was also observed by Cariveau et al. [2014] and Koch and Schmid-Hempel [2011b], and might be explained by a different host ecology or physiology. The bumblebee gut microbiota composition has been reported to be similar over different locations and even between bumblebees sampled in different continents, which indicates a potential functional dependence on the gut microbiota [Cariveau et al., 2014; Koch and Schmid-Hempel, 2011b].

### 5.2.1 Defense against pathogens

The infection success of a specific parasite genotype in a particular host is determined by the host genotype but can also be determined by the type of microbial community present in the host's gut [Koch and Schmid-Hempel, 2012, 2011a]. Gut symbionts can form intimate associations with their hosts and can provide an extended immune system. The genetic, and therefore functional repertoire of this microbial community might change rapidly due to horizontal gene transfer with novel micro-organisms passing through the gut [Koch and Schmid-Hempel, 2012]. This is an important aspect which might enhance the resistance of the host to rapidly evolving parasites.

Gut symbionts can mediate host protection through different mechanisms like stimulation of the immune system, formation of biofilms and growth promotion of other gut symbionts (Figure 5.3). In honeybees the gut microbiota stimulates the immune system resulting in higher levels of antimicrobial peptides in the gut lumen and haemolymph [Kwong et al., 2017]. In addition, most members of the gut microbiota show elevated resistance against these AMPs compared to micro-organisms which are not associated with the bee gut [Kwong et al., 2017]. *Snodgrassella* strains form biofilms in the bumblebee hindgut, which is also the infection site of *C. bombi*, and occur in close proximity with *Gilliamella* strains [Engel et al., 2012; Martinson et al., 2012]. Therefore, competitive exclusion through efficient colonization of the epithelium might explain the negative associations which have been observed between the presence of these bacteria and the parasite *C. bombi* [Cariveau et al., 2014]. *Snodgrassella* and *Gilliamella* might also stimulate each other's growth as *Snodgrassella* oxidises carboxylates produced by *Gilliamella* and *Gilliamella* might obtain essential nutrients like pyrimidines from *Snodgrassella* [Kwong et al., 2014]. Growth promotion of gut symbionts was also observed by a *Fructobacillus* strain *in vitro* [Rokop et al., 2015].

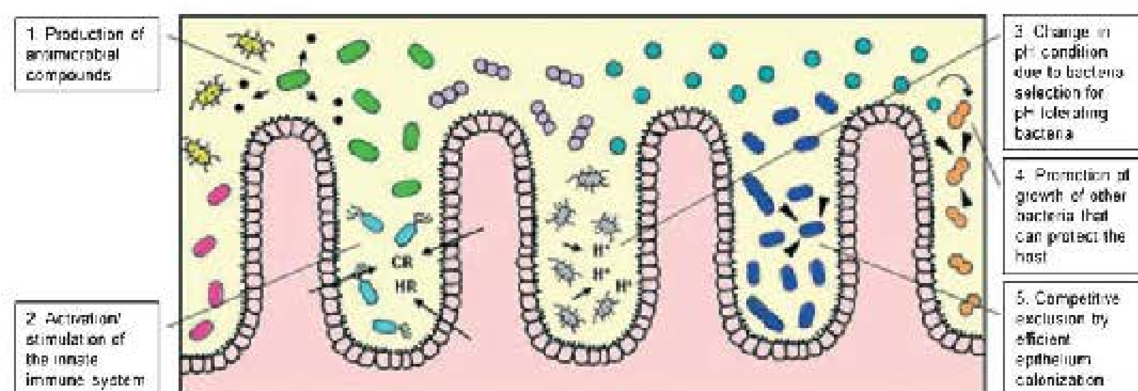


**Figure 5.3:** Mechanisms of gut symbionts to mediate host protection against pathogens. Figure from Hamdi et al. [2011].

### Antimicrobial activity against *C. bombi*

Gut symbionts can inhibit pathogen growth directly by competition for nutrients, by lowering the pH and by the production of antimicrobial compounds (Figure 5.3). The infection success of different *C. bombi* genotypes and the specific immune phenotypes observed largely depend on variations of the gut microbiota [Koch and Schmid-Hempel, 2012]. The mechanism behind this specificity, however, remains largely unknown and might be caused by the production of antimicrobial compounds by gut symbionts. In wild *Anopheles* mosquitoes resistance against *Plasmodium* parasites might at least partially be explained by the presence of a gut symbiont capable to inhibit *Plasmodium* growth *in vivo* through the production of reactive oxygen species [Cirimotich et al., 2014]. To investigate if bumblebee gut micro-organisms can produce anti-trypanosomal compounds and protect bumblebees against *C. bombi* infections, a 96-well inhibition assay was developed in the present PhD study. The supernatant of only six isolates (all *W. bombi* [n = 4], *W. viridescens* [n = 1] and *S. albidoflavus* [n = 1] isolates) inhibited growth of the *C. bombi* strains (Chapter 3). Treatment of the supernatant with catalase, proteinase K or trypsin indicated that the inhibitory activity of the supernatant of the *Weissella* isolates was likely caused by the production of hydrogen peroxide. The inhibition caused by *S. albidoflavus* R-53649 was not neutralised by any of the above mentioned treatments and its mechanism of action remained unidentified. *Streptomyces* species are spore-forming bacteria which are known for the production of bioactive secondary metabolites such as antivirals, antifungals and antibiotics [de Lima Procópio et al., 2012]. The production of these compounds is often species specific [de Lima Procópio et al., 2012] and several antimicrobial compounds from *S. albidoflavus* have been described (e.g. the sesquiterpene albaflavenone, the polyketide antimycin A18 and a straight-chain polyhydroxy, polyether, non-proteinaceous compound with antifungal activity) [Augustine et al., 2005; Guertler et al., 1994; Yan et al., 2010]. We discovered multiple polyketide, terpene, bacteriocin, non-ribosomal peptide and lantipeptide biosynthetic gene clusters in the genome of *S.*

*albidoflavus* R-53649. Based on the active compound's resistance against proteinase K and trypsin, it is likely not proteinaceous; however some non-ribosomal peptides, lantipeptides and bacteriocins, are reported to be protease-resistant as well [Moreno et al., 2000; Nolan and Walsh, 2009; Ouzari et al., 2008]. Chromatography and mass spectrometry analyses should be performed to verify if culture supernatants comprise known secondary metabolites [Lu et al., 2014]. If not, the structure of the compound may be elucidated by first purifying the compound through fractionation of the supernatant by liquid-liquid partitioning and chromatographic separation and subsequently identifying the compound by NMR and mass spectrometry [Augustine et al., 2005].

*W. bombi*, *W. viridescens* and *S. albidoflavus* were only isolated from a few bumblebees and are therefore likely bacteria with a low prevalence in the bumblebee gut or passers-by taken up from the environment. A general misconception is that a probiotic strain should be able to colonise and proliferate in the gut [Fontana et al., 2013]. However, evidence for adherence of probiotic strains to the gut is often based on *in vitro* experiments which do not reflect the *in vivo* situation in which the strains interact with a complex microbial environment and with the host [Fontana et al., 2013; Sanders, 2008]. In addition, many probiotic strains only persist briefly after administration is stopped and are not members of the common gut microbiota of the host (e.g. *Saccharomyces boulardii* and *Bifidobacterium animalis* as human probiotics) [Fontana et al., 2013; Sanders, 2008; Frese, 2012]. The effectiveness of a probiotic strain also depends on the mode and frequency of administration and the dose used. Therefore, *in vivo* experiments are necessary to confirm the beneficial effect of the potentially probiotic strains, to reveal long-term effects and safety for the host and to determine the effective dose and administration approach.

**Antimicrobial activity against *P. larvae*, *M. plutonius* and *A. apis***

*In vitro* and *in vivo* antimicrobial activity against *P. larvae* and *M. plutonius* has been observed for several LAB isolates from the honey crop [Forsgren et al., 2009; Killer et al., 2013; Vasquez et al., 2012]. American and European foulbrood caused by *P. larvae* and *M. plutonius*, respectively, are the most devastating bacterial diseases of honeybees [De Graaf et al., 2006]. As the honeybee gut microbiota is similar to that of bumblebees, microbial isolates from the bumblebee gut with activity against honeybee pathogens might also be good candidates for honeybee probiotics. In the present study a well diffusion assay was performed to analyze the capacity of bumblebee gut isolates to inhibit growth of these foulbrood pathogens. In addition to the type strains of *P. larvae* and *M. plutonius*, two additional *P. larvae* strains were included in the analyses which correspond to the Enterobacterial Repetitive Intergenic Consensus (ERIC) genotypes I (R-54592) and II (R-54334) and differ in virulence [Genersch, 2010]. Most of the tested isolates with activity against foulbrood pathogens were LAB and growth inhibition was only observed when live cells were present. The inhibitory effect was likely not caused by a pH effect and might be explained by nutrient competition or by a higher concentration of antimicrobial compounds when isolates grow in agar wells compared to broth medium. Some antimicrobial compounds are also unstable

in liquid solutions [Le Breton et al., 2007] and the presence of such compounds might be confirmed by an extraction from the inhibition zone agar [Mahenthiralingam et al., 2011]. As it is shown in Chapter 3 that both pathogens are sensitive to lactic and acetic acid, a potential higher concentration of SCFA produced by isolates in the wells compared to broth medium might also cause the absence of pathogen growth. Although there were no isolates of which the supernatant showed activity against the pathogens, nutrient competition and the production of SCFA might be important to compete with pathogens *in vivo*.

We also tested if the isolates were capable of inhibiting growth of the fungal pathogen *A. apis*. *Ascosphaera* causes chalkbrood disease in larvae of social and solitary bees and vegetative and reproductive stages of the fungus have been detected in bumblebee adults [Maxfield-Taylor et al., 2015]. Only one isolate, *C. intestini* R-53529, was able to inhibit growth of *A. apis in vitro*. Growth of this fungal pathogen was also inhibited by 1 M acetic acid and *C. intestini* R-53529, which is an acetic acid bacterium that potentially inhibits growth of the pathogen by the production of acetic acid.

In addition to a well diffusion assay, an overlay assay was performed (Annex Table 1 and 2) because the production of antimicrobial compounds might be different for some strains when grown on solid instead of in liquid medium [Polak-Berecka et al., 2010]. In this assay, five µl of a broth culture of the isolates was spotted in the middle of an AC agar plate and the isolate was allowed to grow for two days. One hundred microliters of pathogen suspension (as described in Chapter 3) was added to 2.5 ml AC agar at pH 7 containing 0.7% agar and this suspension was subsequently poured onto the plate containing the test isolate. Also with this assay several isolates were obtained which inhibited growth of *P. larvae* and *M. plutonius*, and *A. apis* growth was inhibited by *C. intestini* R-53529. However, the inhibition was again lost when supernatant was used instead of live cells. Differences were observed between both assays which might indicate the influence of growth conditions on pathogen growth inhibition e.g. *B. intestini* only inhibited growth of *P. larvae* in the well diffusion assay while the inverse was observed for *B. commune*.

### 5.2.2 Detoxification and nutrient provisioning

Only 20% of the pollen present in the bee rectum is empty which indicates its inefficient digestion by bees [Crailsheim et al., 1992; Szolderits and Crailsheim, 1993]. The pollen wall remains mostly intact while passing through the bee gut and nutrients are presumably released by pollen pseudo-germination and bee digestive enzymes [Roulston and Cane, 2000]. When pollen was incubated in physiological water for two days, we observed that the pollen indeed remained mostly intact (Figure 5.4). However, in some pollen grains the cytoplasm started to protrude through the pollen apertures (thinner regions in the pollen wall) likely indicating germination of the pollen. When pollen was incubated in physiological water acidified to pH 3 or 5, broken pollen walls were observed and it is possible that passage of pollen through the acidic honey stomach also mediates the release of nutrients from pollen.

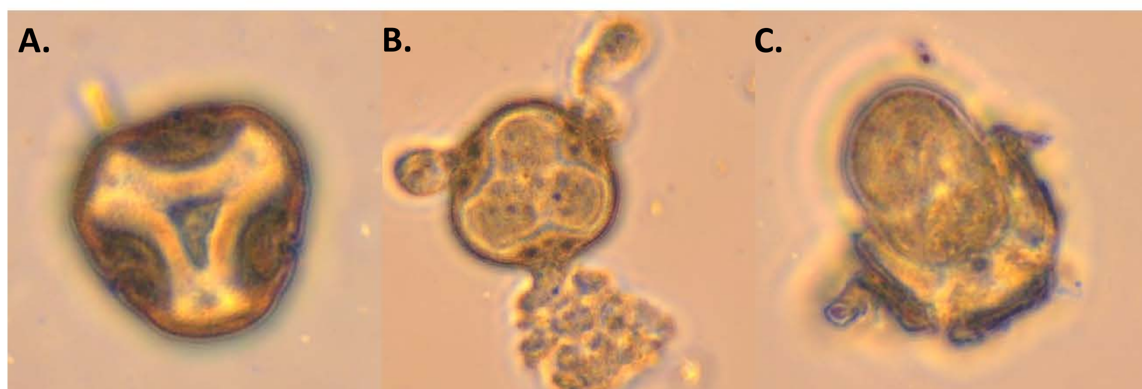


**Figure 5.4:** Pollen incubated in a physiological suspension for two days. A = intact pollen. B. = pollen germination. C = pollen at pH 3 with broken pollen wall.

As described in Chapter 1, gut micro-organisms often contribute to nutrient digestion. The bee gut microbiota potentially contributes to pollen digestion by weakening of the pollen wall and might provide additional nutrients through degradation of undigested compounds like pectin. Pectin is a compound of the pollen wall which is not digested by bees and is toxic to bees [Engel et al., 2012; Zheng et al., 2016]. The hindgut is covered with the intima and nutrient absorption mainly takes place in the midgut. However, the ileum is efficient in absorbing water and ions [Santos and Serrao, 2006]. Undigested pectin or polygalacturonic acid which is highly negatively charged can bind with positively charged ions like the minerals $Mg^{2+}$, $Na^{+}$ and $K^{+}$. These minerals are therefore chelated by pectin which might prevent there efficient uptake and might result in their excretion through defecation [Eliaz et al., 2006].

Genome sequence analysis of different *Gilliamella* strains indicated their potential to ferment pectin, which was confirmed for some strains through *in vitro* experiments [Engel et al., 2012; Zheng et al., 2016]. In the course of our study, we isolated four novel *Gilliamella* species. Although these isolates did not degrade pectin from citrus peel or polygalacturonic acid when grown on MP-7 medium or on AC agar with polygalacturonic acid, a complete pathway for pectin degradation was detected in the genome of LMG 28359$^{T}$. In addition, the genome of LMG 29880$^{T}$ contains the *exuT* gene which codes for a hexuronate transporter that is responsible for transporting D-galacturonate into the cell, and all genes for further degradation of D-galacturonate to pyruvate and glyceraldehyde-3-phosphate. The discrepancy between the observed genotypes and phenotypes may be explained by unsuitable physico-chemical conditions.

Of all the obtained bumblebee gut isolates in the present study, only *B. licheniformis* R-53713 was able to degrade pectin *in vitro* and a complete pectin degradation pathway was present in its genome. Indeed, *B. licheniformis* is known to produce pectate lyases, pectin lyases and polygalacturonases [Rehman et al., 2012; Remoroza et al., 2015]. The isolate exclusively

degraded pectin at pH 7 and PGA at pH 5 which might be explained by a different pH optimum of the enzymes involved in pectin and PGA degradation [Rehman et al., 2012; Remoroza et al., 2015]. Because *B. licheniformis* was only isolated from one bumblebee, this species was likely obtained from the environment. However, bacteria with a low abundance and prevalence in the bumblebee gut might also be important in colony health. If the colony is founded in a region with low food availability, these bacteria may contribute to the degradation of undigested compounds like pectin and thereby the release of additional nutrients.
Few microbial isolates seem to mediate the digestion of pectin and it is therefore likely that the digestion of this compound does not contribute much to the provisioning of the bee with additional nutrients or the weakening of the pollen wall. It is also possible that the interaction of gut microbionts rather than individual strains mediates the release of nutrients from pollen.

A metagenomics study of the honeybee gut microbiota revealed that especially *Bacillus* spp. but also *Bifidobacteriaceae* and members of Beta- and Gammaproteobacteria contain genes encoding glycoside hydrolases with a function in the degradation of plant-derived macromolecules like starch, pectin, glycogen and cellulose [Lee et al., 2015]. *Fructobacillus* spp. might also contribute to pollen digestion as a *Fructobacillus* strain isolated from the honeybee gut was able to degrade the complex pollen compound lignin [Alberoni et al., 2016].

In accordance with the analyses performed by Zheng et al. [2016], we screened the genomes of our *Gilliamella* strains for genes encoding enzymes necessary for the catabolism of xylose, arabinose, rhamnose and mannose which are toxic sugars in nectar. Neither of the four strains contained genes for xylose and rhamnose catabolism and only strain LMG 28359$^{T}$ has the genes to catabolize mannose. The genes *araA*, *araB*, *araD* and *araT* which encode enzymes for the transport of arabinose into the cell and catabolism of arabinose to xylulose-5-phosphate were detected in the genome of LMG 29880$^{T}$.

Fat-soluble vitamins and the amino acids phenylalanine and tryptophane are often lacking in pollen. Analysis of the genome of the *A. mensalis* type strain revealed that bumblebees might obtain additional nutrients like vitamins and amino acids from its gut microbiota.

## 5.3 General conclusions and future perspectives

In the present PhD study, cultivated representatives of most of the detected and previously reported phylotypes were obtained and a more accurate identification was provided. In addition, a MALDI-TOF MS database was established which can facilitate microbial identification during subsequent isolation campaigns focusing on the bumblebee gut. Data presented in chapter 3 showed that the species diversity as revealed through 16S rRNA amplicon sequencing is underestimated as several phylotypes consisted of multiple species and several species, some of which were novel, were isolated from the bumblebee gut for the

first time. No cultivated representatives were obtained for two phylotypes (i.e. Gamma-II and Lacto-IV) and representative isolates might be obtained in the future by providing a set of different media and media containing charcoal to reduce the exposure to toxic compounds or by performing co-culture experiments with bacteria commonly present in the bumblebee gut. Yeast species were also isolated and seem highly prevalent in the bumblebee gut.

Several isolates showed activity against bee pathogens and one isolate was obtained which was capable to degrade pectin *in vitro*. The mechanisms of the observed pathogen inhibition remain largely speculative. Also the active compound of *S. albidoflavus* R-53649 remained unknown and should be determined by structure analyses based on mass spectrometry and/or NMR.

In the course of the present study, the genome was sequenced of one isolate of each of the isolated bacterial species (data were only partially integrated in chapters 3 and 4). Mining of these genomes may provide further insight into the gut symbionts' contribution to the immune system and pathogen defense of bumblebees, and to the detoxification of toxic compounds like pesticides and herbicides. It will also allow a more accurate identification of the isolates which were only identified by 16S rRNA gene sequence analysis in the present study.

As stated above, the potential beneficial effect of candidate probiotic strains should be confirmed through *in vivo* trials. Koch and Schmid-Hempel [2011a] observed significantly reduced levels of the parasite *C. bombi* in bumblebees fed with feces of healthy nest mates. This knowledge can be a guide for the development of an *in vivo* test in which the beneficial effect of the isolates with activity against *C. bombi* can be analyzed. Are bumblebees more resistant to pathogens when they are fed with monocultures of probiotic strains or is a mixture of probiotic strains necessary to observe a beneficial effect? Are these effects comparable to the effect observed when bees are fed with feces? Answering these questions will not only facilitate the application of probiotics in bumblebees but will also provide deeper knowledge into pathogen defense in bumblebees and interactions between bumblebee gut micro-organisms. A potential experimental set-up could be a cross-over experiment as indicated in Figure 5.5. In this set-up one group of bumblebees is fed with the probiotic strain, or strains, for a specific period while another group is only fed sugar water and pollen. After infection with *C. bombi* a cross-over between the groups can be implemented. The infection level typically peaks after 7 days [Koch and Schmid-Hempel, 2011a]. At this point the gut of the bumblebees can be dissected and the infection success can be detected by a *C. bombi* specific qPCR analysis [Koch and Schmid-Hempel, 2011a]. This set-up will indicate if a beneficial effect is observed and if it is necessary to feed the probiotic strains 1) before the infection, 2) after the infection or 3) before and after the infection.

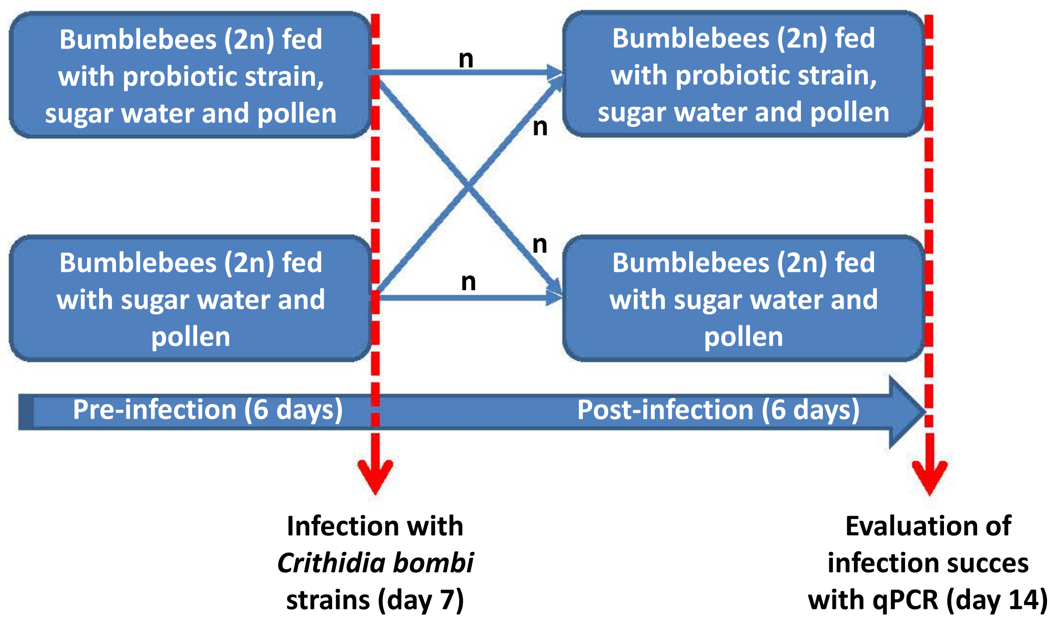


**Figure 5.5:** Experimental set-up for *in vivo* trials.

# Part V

# Bibliography

# Bibliography

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# Curriculum Vitae

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| | |
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## Work Experience

2013-2017 **Doctor of Science: Biochemistry and Biotechnology**

Laboratory of Microbiology (Faculty of Sciences), Ghent University

Implementing a diverse range of molecular techniques for the isolation, detection, identification and description of bacteria and optimizing *in vitro* assays for the functional analysis of bacteria

Presenting at conferences and several lab seminars, writing and publishing peer-reviewed scientific articles

Supervisor of several master and bachelor students and assistant in practical courses at the Faculty of Sciences, Ghent University

August 2012 **Internship Prodigest**

Technologiepark Zwijnaarde 607, 9052 Ghent

fatty acid analyses, ammonia analyses and DNA-extraction

## Education

2013-2017 **Doctor of Science: Biochemistry and Biotechnology**

Laboratory of Microbiology (Faculty of Sciences), Ghent University

Dissertation: The gut microbiota of bumblebees: a treasure chest of biodiversity and functionality

Promotors: Peter Vandamme and Guy Smagghe
Training: Bionumerics, MEGA6, PAST, Rstudio, RAST online annotation tool and CLCgenomicswb7

2013-2017 **Doctoral training programme**

Doctoral schools transferable skills courses: advanced academic English writing skills, advanced academic presentation skills in English and personal effectiveness.

Doctoral schools specialist courses: BITS LINUX training, Data visualisation for Biology: a practical workshop on design, techniques and tools and Statistics –Basics of Statistical Inference. Extra courses: introduction to R.

2011-2013 **Master of Science in Biochemistry and Biotechnology**

Specialization: Microbial Biotechnology Ghent University
Dissertation: 'The environmental niche of *Burkholderia multivorans*: a reservoir for a cystic fibrosis pathogen?'
Promoter: Prof. Dr. Peter Vandamme

2008-2011 **Bachelor of Science in Biochemistry and Biotechnology**

Ghent University

# Social and organizational skills

- Active participation in outreach activities and science popularization ('Dag van de Wetenschap' 2013, 2014, 2015 and 2016) and assistant at the International Tuberculosis conference (March 21st 2014, Het Pand Ghent).
- Coordination of practical course Microbiology of 3rd Bachelor in Biochemistry and Biotechnology
- Supervision of master thesis students (Steven Goossens from September to May 2015 and Simon Felix from September to May 2016) and high school students (4 day internship in November 2014 (Jochen Sansen) and 1 week internship in January 2016 (Annelies Van Parys, Johannes Gouwy and James De Lille)).

# Computer skills

Rstudio, PAST, Bionumerics, Microsoft Office, Latex, MEGA6, CLCgenomicswb7 and RAST

# Poster and Oral presentations

- Poster presentation at the BISMiS 2014 meeting in Edinburgh, Scotland (April 7th-11th 2014).
- BSM conferences, Academy Palace, Brussels: 'Microbial Diversity for Science and Industry' (2013), 'Cell signalling –host –microbiome interactions' (2014), 'Microbes and the global change' (2015) and 'Microbiome and host metabolism' (2016).
- Poster presentation at 'Dag van de wetenschap' (2014 and 2015).
- Poster presentation at the IPC 2015 meeting in Budapest, Hungary (June 23th-25th 2015).
- Oral presentation of 30 minutes at the mini-symposium of the Flemish research consortium on fermented foods and beverages on April 2th, 2015 (Food fermentations: taxonomy and physiology of microorganisms involved) and March 24th, 2016 (Microbial food ecosystems).

- Lab seminars: oral presentations (November 2013 and April and September 2014) at the laboratory of microbiology (LM-UGent).
- Oral presentation at the Eurbee7 conference (section Pathology) in Cluj–Napoca, Romania (September 7th–9th, 2016)

## Scientific publications

2015 | L. Li, J. Praet, W. Borremans, O. C. Nunes, C. M. Manaia, I. Cleenwerck, I. Meeus, G. Smagghe, L. De Vuyst, and P. Vandamme. 2015. *Bombella intestini* gen. nov., sp. nov., a novel acetic acid bacterium isolated from the bumblebee crop. Int. J. Syst. Evol. Microbiol. 65:267-275.

2015 | J. Praet, I. Meeus, M. Cnockaert, M. Aerts, G. Smagghe, and P. Vandamme. 2015. *Bifidobacterium commune* sp. nov. isolated from the bumblebee gut. Antonie van Leeuwenhoek DOI :10.1007/s10482-015-0425-3.

2015 | J. Praet, I. Meeus, M. Cnockaert, K. Houf, G. Smagghe, and P. Vandamme. 2015. Novel lactic acid bacteria isolated from the bumblebee gut: *Convivina intestini* gen. nov., sp. nov., *Lactobacillus bombicola* sp. nov., and *Weissella bombi* sp. nov. Antonie van Leeuwenhoek DOI :10.1007/s10482-015-0429-z.

2016 | J. Praet, M. Aerts, E. De Brandt, I. Meeus, G. Smagghe, and P. Vandamme. 2016. *Apibacter mensalis* sp. nov.: a rare member of the bumblebee gut microbiota Int J Syst Evol Microbiol. doi: 10.1099/ijsem.0.000921

2017 | J. Praet, M. Cnockaert, I. Meeus, G. Smagghe, P. Vandamme (2017). *Gilliamella intestini* sp. nov., *Gilliamella bombicola* sp. nov., *Gilliamella bombi* sp. nov. and *Gilliamella mensalis* sp. nov.: four novel *Gilliamella* species isolated from the bumblebee gut. Systematic and Applied Microbiology, 2017.

2017 | J. Praet, A. Parmentier, R. Schmid-hempel, I. Meeus, G. Smagghe, and P. Vandamme. Large-scale cultivation of the bumblebee gut microbiota reveals an underestimated bacterial species diversity and functionality. In preparation.

## Additional information

Personality: eager to learn, motivated team-player

Languages: Dutch (mother tongue), English (proficiant user), French (basic user)

Other: Driver's license B

# Part VI

# Annex

# Annex

**Annex 1**. Antimicrobial activity of bumblebee gut isolates determined by an overlay assay.

| Phylum | Species | Number of tested isolates | *P. larvae* LMG 9820$^T$ | *P. larvae* R-54592 | *P. larvae* R-54334 | *M. plutonius* LMG 20360$^T$ |
|---|---|---|---|---|---|---|
| Actinobacteria | *Bifidobacterium actinocoloniforme* | 2 | 0 | 0 | 0 | 0 |
| | *Bifidobacterium bombi* | 2 | 2 | 2 | 2 | 2 |
| | *Bifidobacterium commune* | 5 | 5 | 5 | 5 | 0 |
| | *Bombiscardovia coagulans* | 21 | 0 | 0 | 0 | 0 |
| | *Kocuria rhizophila* | 2 | 1 | 1 | 1 | 0 |
| | *Micrococcus luteus* | 1 | 0 | 0 | 0 | 0 |
| | *Streptomyces albidoflavus* | 1 | 0 | 0 | 0 | 0 |
| Alphaproteobacteria | *Bombella intestini* | 1 | 0 | 0 | 0 | 0 |
| | *Commensalibacter intestini* | 1 | 1 | 1 | 1 | 0 |
| Betaproteobacteria | *Snodgrassella alvi* | 20 | 0 | 0 | 0 | 0 |
| Gammaproteobacteria | *Enterobacter kobei* | 1 | 0 | 0 | 0 | 0 |
| | *Gilliamella bombi* | 1 | 0 | 0 | 0 | 0 |
| | *Gilliamella bombicola* | 2 | 0 | 0 | 0 | 0 |
| | *Gilliamella intestini* | 1 | 0 | 0 | 0 | 0 |
| | *Gilliamella mensalis* | 1 | 0 | 0 | 0 | 0 |
| | *Hafnia alvei* | 8 | 5 | 5 | 5 | 1 |
| | *Moraxella osloensis* | 1 | 0 | 0 | 0 | 0 |
| | *Rosenbergiella nectareae* | 1 | 0 | 0 | 0 | 0 |
| | *Serratia marcescens* | 2 | 0 | 0 | 0 | 1 |
| Bacteroidetes | *Apibacter mensalis* | 2 | 0 | 0 | 0 | 0 |
| Firmicuts | *Bacillus licheniformis* | 1 | 0 | 0 | 0 | 0 |
| | *Bacillus spp.* | 1 | 0 | 0 | 0 | 0 |
| | *Convivina intestini* | 3 | 3 | 3 | 3 | 1 |
| | *Enterococcus faecalis* | 3 | 3 | 3 | 3 | 3 |
| | *Fructobacillus fructosus* | 24 | 18 | 18 | 18 | 7 |
| | *Fructobacillus tropeaoli* | 16 | 13 | 13 | 13 | 1 |
| | *Lactobacillus apis* | 2 | 2 | 2 | 2 | 2 |
| | *Lactobacillus bombi* | 2 | 1 | 0 | 1 | 0 |
| | *Lactobacillus bombicola* | 38 | 34 | 33 | 34 | 23 |
| | *Lactobacillus kimchicus* | 5 | 5 | 5 | 5 | 4 |
| | *Lactobacillus kunkeei* | 13 | 6 | 7 | 5 | 1 |
| | *Lactococcus lactis* | 10 | 6 | 6 | 6 | 2 |
| | *Leuconostoc mesenteroides* | 1 | 1 | 1 | 1 | 1 |
| | *Leuconostoc pseudomesenteroides* | 3 | 3 | 3 | 3 | 3 |
| | *Leuconostoc* sp. | 1 | 0 | 0 | 0 | 0 |
| | *Lysinibacillus fusiformis* | 8 | 0 | 0 | 0 | 0 |
| | *Paenibacillus barengoltzii* | 1 | 0 | 0 | 0 | 0 |
| | *Ureibacillus suwonensis* | 1 | 0 | 0 | 0 | 0 |
| | *Vagococcus entomophilus* | 1 | 1 | 1 | 1 | 1 |
| | *Weissella bombi* | 5 | 5 | 5 | 5 | 5 |
| | *Weissella viridescens* | 1 | 1 | 1 | 1 | 1 |
| Fungi | *Candida* sp. I | 4 | 0 | 0 | 0 | 0 |
| | *Candida* sp. II | 1 | 0 | 0 | 0 | 0 |
| | *Candida bombi* | 8 | 0 | 0 | 0 | 0 |
| | *Candida bombiphila* | 2 | 0 | 0 | 0 | 0 |
| | *Zygosaccharomyces rouxii* | 2 | 0 | 0 | 0 | 0 |
| | | 233 | 116 | 115 | 115 | 59 |

**Annex 2**. Antimicrobial activity of all tested bumblebee gut isolates.

| | OL *P. larvae* LMG 9820$^T$ | OL *P. larvae* R-54592 | OL *P. larvae* R-54334 | OL *M. plutonius* LMG 20360$^T$ | WD P. larvae LMG 9820$^T$ | WD P. larvae R-54592 | WD P. larvae R-54334 | WD M. plutonius LMG 20360$^T$ |
|---|---|---|---|---|---|---|---|---|
| *Bifidobacterium actinocoloniforme* R-53514 | - | - | - | - | - | - | - | - |
| *Bifidobacterium actinocoloniforme* R-53049 | - | - | - | - | - | - | - | - |
| *Bifidobacterium bombi* R-54661 | +++ | +++ | ++ | - | ++ | + | + | - |
| *Bifidobacterium bombi* R-54689 | +++ | +++ | +++ | - | ++ | ++ | + | - |
| *Bifidobacterium commune* R-52791 | ++ | +++ | +++ | - | - | - | - | - |
| *Bifidobacterium commune* R-53521 | ++ | +++ | +++ | - | - | - | - | - |
| *Bifidobacterium commune* R-55213 | +++ | ++ | ++ | - | - | - | - | - |
| *Bifidobacterium commune* R-53124 | ++ | +++ | +++ | - | - | - | - | - |
| *Bifidobacterium commune* R-54652 | ++ | +++ | +++ | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53558 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53711 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53714 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53721 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53729 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-54642 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-55224 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-55251 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-55257 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-54672 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-54679 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-54682 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-54684 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-54869 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-55279 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-55202 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53126 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53128 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53129 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53130 | - | - | - | - | - | - | - | - |
| *Bombiscardovia coagulans* R-53133 | - | - | - | - | - | - | - | - |
| *Kocuria rhizophila* R-54663 | ++ | +++ | ++ | - | - | - | - | - |
| *Kocuria rhizophila* R-55281 | - | - | - | - | - | - | - | - |
| *Micrococcus luteus* R-53632 | - | - | - | - | - | - | - | - |
| *Streptomyces albidoflavus* R-53649 | - | - | - | - | - | - | - | - |
| *Bombella intestini* R-54242 | - | - | - | - | ++ | ++ | ++ | - |
| *Commensalibacter intestini* R-53529 | ++ | + | ++ | - | ++ | ++ | ++ | - |
| *Snodgrassella alvi* R-53633 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53634 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53656 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53583 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53503 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53523 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53528 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53680 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-53677 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-54674 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-54695 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-54841 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-54858 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-54863 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-55215 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-55223 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-55243 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-55249 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-54236 | - | - | - | - | - | - | - | - |
| *Snodgrassella alvi* R-55254 | - | - | - | - | - | - | - | - |
| *Enterobacter kobei* R-54666 | - | - | - | - | - | - | - | - |
| *Gilliamella bombi* R-54662 | - | - | - | - | - | - | - | - |
| *Gilliamella bombicola* R-53248 | - | - | - | - | - | - | - | - |
| *Gilliamella bombicola* R-53705 | - | - | - | - | - | - | - | - |
| *Gilliamella intestini* R-53144 | - | - | - | - | - | - | - | - |
| *Gilliamella mensalis* R-54852 | - | - | - | - | - | - | - | - |
| *Hafnia alvei* R-53631 | +++ | +++ | +++ | ++ | +++ | ++ | +++ | ++ |
| *Hafnia alvei* R-54239 | ++ | +++ | ++ | - | ++ | ++ | ++ | - |
| *Hafnia alvei* R-53736 | +++ | +++ | +++ | - | ++ | ++ | ++ | - |
| *Hafnia alvei* R-53673 | ++ | +++ | ++ | - | + | + | - | - |
| *Hafnia alvei* R-55238 | ++ | +++ | +++ | - | ++ | + | - | - |
| *Hafnia alvei* R-54855 | - | - | - | - | - | - | - | - |
| *Hafnia alvei* R-53703 | - | - | - | - | - | - | - | - |
| *Hafnia alvei* R-53704 | - | - | - | - | - | - | - | - |
| *Moraxella osloensis* R-53657 | - | - | - | - | - | - | - | - |
| *Rosenbergiella nectareae* R-53659 | - | - | - | - | - | - | - | - |
| *Serratia marcescens* R-53686 | - | - | - | - | + | + | + | - |
| *Serratia marcescens* R-54856 | - | - | - | ++ | ++ | ++ | ++ | ++ |
| *Apibacter mensalis* R-53146 | - | - | - | - | - | - | - | - |
| *Apibacter mensalis* R-53136 | - | - | - | - | - | - | - | - |
| *Bacillus licheniformis* R-53713 | - | - | - | - | - | - | - | - |
| *Bacillus* sp. R-53683 | - | - | - | - | - | - | - | - |
| *Convivina intestini* R-54250 | ++ | ++ | ++ | - | ++ | ++ | ++ | - |
| *Convivina intestini* R-53105 | ++ | ++ | +++ | ++ | +++ | ++ | ++ | ++ |
| *Convivina intestini* R-53106 | +++ | ++ | ++ | +++ | +++ | ++ | ++ | ++ |
| *Enterococcus faecalis* R-53509 | ++ | +++ | ++ | ++ | +++ | ++ | ++ | ++ |
| *Enterococcus faecalis* R-54226 | +++ | +++ | +++ | +++ | +++ | ++ | ++ | ++ |
| *Enterococcus faecalis* R-55230 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ |

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| | OL *P. larvae* LMG 9820$^T$ | OL *P. larvae* R-54592 | OL *P. larvae* R-54334 | OL *M. plutonius* LMG 20360$^T$ | WD P. larvae LMG 9820$^T$ | WD P. larvae R-54592 | WD P. larvae R-54334 | WD M. plutonius LMG 20360$^T$ |
|---|---|---|---|---|---|---|---|---|
| *Fructobacillus fructosus* R-54832 | +++ | +++ | +++ | ++ | + | ++ | ++ | - |
| *Fructobacillus fructosus* R-54839 | ++ | +++ | +++ | ++ | + | ++ | ++ | - |
| *Fructobacillus fructosus* R-54842 | +++ | +++ | +++ | ++ | ++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-54245 | +++ | +++ | +++ | ++ | ++ | ++ | +++ | - |
| *Fructobacillus fructosus* R-54251 | +++ | +++ | +++ | ++ | +++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-54654 | +++ | +++ | +++ | ++ | +++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-55210 | +++ | ++ | ++ | + | +++ | +++ | ++ | - |
| *Fructobacillus fructosus* R-54382 | +++ | ++ | +++ | - | ++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-53694 | +++ | +++ | +++ | - | +++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-53688 | +++ | +++ | +++ | - | ++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-54664 | +++ | +++ | +++ | - | +++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-53138 | +++ | +++ | +++ | - | +++ | ++ | +++ | - |
| *Fructobacillus fructosus* R-53501 | +++ | +++ | +++ | - | +++ | ++ | +++ | - |
| *Fructobacillus fructosus* R-53525 | +++ | +++ | +++ | - | +++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-54232 | +++ | +++ | +++ | - | ++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-54247 | +++ | +++ | +++ | - | +++ | ++ | +++ | - |
| *Fructobacillus fructosus* R-54249 | ++ | +++ | +++ | - | +++ | +++ | +++ | - |
| *Fructobacillus fructosus* R-54675 | +++ | +++ | +++ | - | ++ | ++ | ++ | - |
| *Fructobacillus fructosus* R-54861 | - | - | - | - | - | - | - | - |
| *Fructobacillus fructosus* R-54857 | - | - | - | - | - | - | - | - |
| *Fructobacillus fructosus* R-55212 | - | - | - | - | - | - | - | - |
| *Fructobacillus fructosus* R-55225 | - | - | - | - | - | - | - | - |
| *Fructobacillus fructosus* R-55226 | - | - | - | - | - | - | - | - |
| *Fructobacillus fructosus* R-55231 | - | - | - | - | - | - | - | - |
| *Fructobacillus tropeaoli* R-54848 | +++ | +++ | +++ | ++ | + | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-53137 | +++ | +++ | +++ | - | +++ | ++ | +++ | - |
| *Fructobacillus tropeaoli* R-53502 | +++ | +++ | +++ | - | ++ | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-53534 | +++ | +++ | +++ | - | +++ | ++ | +++ | - |
| *Fructobacillus tropeaoli* R-53718 | ++ | ++ | ++ | - | ++ | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-54860 | +++ | +++ | +++ | - | +++ | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-55203 | +++ | +++ | +++ | - | +++ | +++ | ++ | - |
| *Fructobacillus tropeaoli* R-55229 | +++ | + | + | - | +++ | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-55234 | +++ | +++ | +++ | - | ++ | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-55250 | +++ | +++ | +++ | - | +++ | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-53640 | +++ | ++ | +++ | - | +++ | ++ | +++ | - |
| *Fructobacillus tropeaoli* R-53735 | + | ++ | ++ | - | +++ | ++ | ++ | - |
| *Fructobacillus tropeaoli* R-54837 | +++ | +++ | +++ | - | +++ | ++ | +++ | - |
| *Fructobacillus tropeaoli* R-55211 | - | - | - | - | - | - | - | - |
| *Fructobacillus tropeaoli* R-55219 | - | - | - | - | - | - | - | - |
| *Fructobacillus tropeaoli* R-54840 | - | - | - | - | - | - | - | - |
| *Lactobacillus apis* R-53520 | ++ | ++ | +++ | ++ | ++ | ++ | + | ++ |
| *Lactobacillus apis* R-54867 | +++ | ++ | ++ | ++ | +++ | ++ | ++ | ++ |
| *Lactobacillus bombi* R-54649 | - | - | - | - | - | - | - | - |
| *Lactobacillus bombi* R-54686 | + | - | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-53092 | ++ | ++ | ++ | ++ | +++ | ++ | +++ | ++ |
| *Lactobacillus bombicola* R-53107 | +++ | +++ | ++ | +++ | ++ | ++ | ++ | ++ |
| *Lactobacillus bombicola* R-53702 | +++ | +++ | +++ | +++ | ++ | ++ | ++ | ++ |
| *Lactobacillus bombicola* R-53142 | +++ | +++ | +++ | ++ | +++ | ++ | + | + |
| *Lactobacillus bombicola* R-54828 | ++ | ++ | ++ | ++ | ++ | ++ | + | - |
| *Lactobacillus bombicola* R-53241 | +++ | +++ | +++ | ++ | ++ | ++ | - | - |
| *Lactobacillus bombicola* R-53132 | +++ | +++ | +++ | ++ | ++ | +++ | - | - |
| *Lactobacillus bombicola* R-55248 | +++ | ++ | ++ | ++ | +++ | - | - | ++ |
| *Lactobacillus bombicola* R-53554 | +++ | +++ | +++ | ++ | ++ | - | - | ++ |
| *Lactobacillus bombicola* R-53121 | +++ | +++ | +++ | ++ | ++ | - | - | - |
| *Lactobacillus bombicola* R-53708 | +++ | ++ | +++ | ++ | ++ | - | - | - |
| *Lactobacillus bombicola* R-53542 | +++ | +++ | ++ | ++ | + | - | - | - |
| *Lactobacillus bombicola* R-55242 | +++ | ++ | ++ | ++ | - | - | - | - |
| *Lactobacillus bombicola* R-53093 | + | + | ++ | ++ | - | - | ++ | - |
| *Lactobacillus bombicola* R-53738 | +++ | ++ | ++ | - | ++ | ++ | ++ | - |
| *Lactobacillus bombicola* R-53102 | + | + | + | - | + | + | + | - |
| *Lactobacillus bombicola* R-53522 | +++ | +++ | +++ | - | ++ | - | - | - |
| *Lactobacillus bombicola* R-54237 | ++ | ++ | ++ | - | +++ | - | +++ | - |
| *Lactobacillus bombicola* R-54643 | ++ | ++ | ++ | + | +++ | - | + | - |
| *Lactobacillus bombicola* R-54229 | ++ | ++ | ++ | + | - | - | ++ | - |
| *Lactobacillus bombicola* R-53728 | ++ | ++ | ++ | + | - | - | - | - |
| *Lactobacillus bombicola* R-54688 | ++ | ++ | ++ | ++ | - | - | - | - |
| *Lactobacillus bombicola* R-54691 | ++ | ++ | ++ | ++ | - | - | - | - |
| *Lactobacillus bombicola* R-54692 | ++ | ++ | ++ | ++ | - | - | - | - |
| *Lactobacillus bombicola* R-54694 | ++ | ++ | ++ | + | - | - | - | - |
| *Lactobacillus bombicola* R-54838 | ++ | ++ | ++ | + | - | - | - | - |
| *Lactobacillus bombicola* R-54673 | ++ | ++ | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-54833 | ++ | ++ | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-54849 | ++ | ++ | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-54850 | ++ | ++ | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-54853 | ++ | ++ | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-54859 | ++ | ++ | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-55239 | +++ | ++ | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-53109 | ++ | - | ++ | - | - | - | - | - |
| *Lactobacillus bombicola* R-55221 | - | - | - | - | - | - | - | - |
| *Lactobacillus bombicola* R-55255 | - | - | - | - | - | - | - | - |
| *Lactobacillus bombicola* R-53104 | - | - | - | - | - | - | - | - |
| *Lactobacillus bombicola* R-53561 | - | - | - | - | - | - | - | - |

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| | OL *P. larvae* LMG 9820$^T$ | OL *P. larvae* R-54592 | OL *P. larvae* R-54334 | OL *M. plutonius* LMG 20360$^T$ | WD P. larvae LMG 9820$^T$ | WD P. larvae R-54592 | WD P. larvae R-54334 | WD M. plutonius LMG 20360$^T$ |
|---|---|---|---|---|---|---|---|---|
| *Lactobacillus kimchicus* R-53535 | +++ | +++ | ++ | ++ | ++ | +++ | ++ | ++ |
| *Lactobacillus kimchicus* R-53510 | ++ | +++ | +++ | ++ | ++ | + | ++ | ++ |
| *Lactobacillus kimchicus* R-54644 | ++ | ++ | +++ | ++ | - | - | - | - |
| *Lactobacillus kimchicus* R-54687 | ++ | ++ | ++ | ++ | - | - | - | - |
| *Lactobacillus kimchicus* R-53646 | + | +++ | +++ | - | ++ | + | ++ | - |
| *Lactobacillus kunkeei* R-54246 | + | + | - | + | ++ | ++ | ++ | + |
| *Lactobacillus kunkeei* R-53685 | ++ | ++ | +++ | - | +++ | ++ | ++ | - |
| *Lactobacillus kunkeei* R-54645 | ++ | ++ | +++ | - | +++ | ++ | + | - |
| *Lactobacillus kunkeei* R-54665 | + | + | ++ | - | + | ++ | + | - |
| *Lactobacillus kunkeei* R-55216 | +++ | ++ | ++ | - | +++ | ++ | +++ | - |
| *Lactobacillus kunkeei* R-53654 | ++ | ++ | - | - | ++ | ++ | +++ | - |
| *Lactobacillus kunkeei* R-53693 | - | +++ | ++ | - | - | ++ | ++ | - |
| *Lactobacillus kunkeei* R-54829 | - | - | - | - | + | ++ | + | - |
| *Lactobacillus kunkeei* R-54238 | - | - | - | - | - | - | + | - |
| *Lactobacillus kunkeei* R-54248 | - | - | - | - | - | - | - | - |
| *Lactobacillus kunkeei* R-54671 | - | - | - | - | - | - | - | - |
| *Lactobacillus kunkeei* R-55228 | - | - | - | - | - | - | - | - |
| *Lactobacillus kunkeei* R-55244 | - | - | - | - | - | - | - | - |
| *Lactococcus lactis* R-53701 | ++ | ++ | +++ | +++ | ++ | +++ | +++ | + |
| *Lactococcus lactis* R-53122 | +++ | ++ | +++ | +++ | +++ | +++ | +++ | ++ |
| *Lactococcus lactis* R-53719 | +++ | +++ | +++ | - | ++ | ++ | +++ | - |
| *Lactococcus lactis* R-54228 | +++ | +++ | ++ | - | +++ | ++ | +++ | - |
| *Lactococcus lactis* R-53642 | ++ | ++ | ++ | - | ++ | ++ | ++ | - |
| *Lactococcus lactis* R-53658 | ++ | ++ | +++ | - | ++ | ++ | ++ | - |
| *Lactococcus lactis* R-53508 | - | - | - | - | - | - | - | - |
| *Lactococcus lactis* R-54233 | - | - | - | - | - | - | - | - |
| *Lactococcus lactis* R-54667 | - | - | - | - | - | - | - | - |
| *Lactococcus lactis* R-55199 | - | - | - | - | - | - | - | - |
| *Leuconostoc mesenteroides* R-53706 | +++ | ++ | +++ | +++ | +++ | ++ | ++ | - |
| *Leuconostoc pseudomesenteroides* R-53630 | ++ | ++ | +++ | +++ | ++ | ++ | ++ | ++ |
| *Leuconostoc pseudomesenteroides* R-54874 | ++ | +++ | ++ | +++ | ++ | + | + | ++ |
| *Leuconostoc pseudomesenteroides* R-53098 | ++ | +++ | ++ | ++ | +++ | ++ | ++ | ++ |
| *Leuconostoc* sp. R-54876 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-53675 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-54234 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-55253 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-53638 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-53647 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-53648 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-53650 | - | - | - | - | - | - | - | - |
| *Lysinibacillus fusiformis* R-53532 | - | - | - | - | - | - | - | - |
| *Paenibacillus barengoltzii* R-53679 | - | - | - | - | - | - | - | - |
| *Ureibacillus suwonensis* R-53678 | - | - | - | - | - | - | - | - |
| *Vagococcus entomophilus* R-54668 | + | + | + | ++ | ++ | + | +++ | ++ |
| *Weissella bombi* R-53094 | +++ | +++ | +++ | ++ | +++ | +++ | +++ | ++ |
| *Weissella bombi* R-53537 | +++ | +++ | +++ | +++ | +++ | ++ | +++ | + |
| *Weissella bombi* R-54230 | +++ | +++ | +++ | ++ | +++ | +++ | +++ | ++ |
| *Weissella bombi* R-54240 | +++ | +++ | ++ | ++ | +++ | ++ | ++ | ++ |
| *Weissella bombi* R-54243 | +++ | +++ | ++ | ++ | +++ | +++ | +++ | ++ |
| *Weissella viridescens* R-53536 | +++ | +++ | +++ | +++ | +++ | ++ | +++ | ++ |
| *Candida* sp. I R-53533 | - | - | - | - | - | - | - | - |
| *Candida* sp. I R-53674 | - | - | - | - | - | - | - | - |
| *Candida* sp. I R-55246 | - | - | - | - | - | - | - | - |
| *Candida* sp. I R-55209 | - | - | - | - | - | - | - | - |
| *Candida* sp. II R-53526 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-53672 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-55198 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-55201 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-55206 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-55232 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-55236 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-55241 | - | - | - | - | - | - | - | - |
| *Candida bombi* R-55247 | - | - | - | - | - | - | - | - |
| *Candida bombiphila* R-53682 | - | - | - | - | - | - | - | - |
| *Candida bombiphila* R-53684 | - | - | - | - | - | - | - | - |
| *Zygosaccharomyces rouxii* R-55258 | - | - | - | - | - | - | - | - |
| *Zygosaccharomyces rouxii* R-53505 | - | - | - | - | - | - | - | - |

OL = Overlay assay. WD = Well diffussion assay. - = no inhibition, + = $<2$ mm inhibition radius, ++ = $\leq 1$ cm inhibition radius, +++ = $>1$ cm radius.

**Annex 3**. Isolate list.

| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-52790 | *Weissella bombi* | *B. terrestris* | Gentbrugge | 2013 |
| R-52791 (LMG 28292$^T$) | *Bifidobacterium commune* | *B. lapidarius* | Gentbrugge | 2013 |
| R-53049 | *Bifidobacterium actinocoloniiforme* | *B. pascuorum* | Ledeganck | 2013 |
| R-53092 | *Lactobacillus bombicola* | *B. pascuorum* | Bourgoyen | 2013 |
| R-53093 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2013 |
| R-53094 (LMG 28290T) | *Weissella bombi* | *B. terrestris* | Gentbrugge | 2013 |
| R-53095 | *Weissella bombi* | *B. terrestris* | Gentbrugge | 2013 |
| R-53096 | *Weissella bombi* | *B. terrestris* | Gentbrugge | 2013 |
| R-53097 | *Gilliamella intestini* | *B. lapidarius* | Coupure | 2013 |
| R-53098 | *Leuconostoc pseudomesenteroides* | *B. pascuorum* | Coupure | 2013 |
| R-53102 (LMG 28288$^T$) | *Lactobacillus bombicola* | *B. lapidarius* | Bourgoyen | 2013 |
| R-53103 | *Lactobacillus bombicola* | *B. lapidarius* | Bourgoyen | 2013 |
| R-53104 (LMG 28289) | *Lactobacillus bombicola* | *B. pascuorum* | Coupure | 2013 |
| R-53105 (LMG 28291$^T$) | *Convivina intestini* | *B. terrestris* | Bourgoyen | 2013 |
| R-53106 (LMG 28625) | *Convivina intestini* | *B. terrestris* | Bourgoyen | 2013 |
| R-53107 | *Lactobacillus bombicola* | *B. vestalis* | Wetteren | 2013 |
| R-53108 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53109 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53110 | *Lactobacillus bombicola* | *B. lucorum* | Wetteren | 2013 |
| R-53121 | *Lactobacillus bombicola* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53122 | *Lactococcus lactis* | *B. terrestris* | Ledeganck | 2013 |
| R-53123 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53124 | *Bifidobacterium commune* | *B. terrestris* | Ledeganck | 2013 |
| R-53126 | *Bombiscardovia coagulans* | *B. pascuorum* | Coupure | 2013 |
| R-53127 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2013 |
| R-53128 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2013 |
| R-53129 | *Bombiscardovia coagulans* | *B. lapidarius* | Bourgoyen | 2013 |
| R-53130 | *Bombiscardovia coagulans* | *B. lucorum* | Wetteren | 2013 |
| R-53132 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53133 | *Bombiscardovia coagulans* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53134 | *Lactobacillus bombicola* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53135 | *Lactobacillus bombicola* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53136 (LMG 28623) | *Apibacter mensalis* | *B. lapidarius* | Bourgoyen | 2013 |
| R-53137 | *Fructobacillus tropaeoli* | *B. lapidarius* | Coupure | 2013 |
| R-53138 | *Fructobacillus fructosus* | *B. lapidarius* | Coupure | 2013 |
| R-53139 | *Fructobacillus fructosus* | *B. lapidarius* | Coupure | 2013 |
| R-53140 | *Fructobacillus fructosus* | *B. lapidarius* | Coupure | 2013 |
| R-53141 | *Fructobacillus fructosus* | *B. lapidarius* | Coupure | 2013 |
| R-53142 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53143 | *Gilliamella intestini* | *B. lapidarius* | Coupure | 2013 |
| R-53144 (LMG 28358$^T$) | *Gilliamella intestini* | *B. lapidarius* | Coupure | 2013 |
| R-53145 | *Gilliamella intestini* | *B. lapidarius* | Coupure | 2013 |
| R-53146 (LMG 28357$^T$) | *Apibacter mensalis* | *B. lapidarius* | Bourgoyen | 2013 |
| R-53241 | *Lactobacillus bombicola* | *B. lucorum* | Wetteren | 2014 |
| R-53248 (LMG 28359$^T$) | *Gilliamella bombicola* | *B. pascuorum* | Ledeganck | 2014 |
| R-53499 | *Weissella viridescens* | *B. terrestris* | Ledeganck | 2013 |
| R-53500 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |

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| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-53501 | *Fructobacillus fructosus* | *B. terrestris* | Ledeganck | 2013 |
| R-53502 | *Fructobacillus tropaeoli* | *B. terrestris* | Ledeganck | 2013 |
| R-53503 | *Snodgrassella alvi* | *B. terrestris* | Ledeganck | 2013 |
| R-53504 | *Snodgrassella alvi* | *B. terrestris* | Ledeganck | 2013 |
| R-53505 | *Zygosaccharomyces rouxii* | *B. terrestris* | Ledeganck | 2013 |
| R-53506 | *Zygosaccharomyces rouxii* | *B. terrestris* | Ledeganck | 2013 |
| R-53507 | *Fructobacillus fructosus* | *B. terrestris* | Ledeganck | 2013 |
| R-53508 | *Lactococcus lactis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53509 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53510 | *Lactobacillus kimchicus* | *B. terrestris* | Bourgoyen | 2013 |
| R-53511 | *Lactobacillus kimchicus* | *B. terrestris* | Bourgoyen | 2013 |
| R-53512 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53513 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53514 | *Bifidobacterium actinocoloniiforme* | *B. terrestris* | Bourgoyen | 2013 |
| R-53515 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53516 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53517 | *Lactobacillus kimchicus* | *B. terrestris* | Bourgoyen | 2013 |
| R-53518 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53519 | *Lactobacillus kimchicus* | *B. terrestris* | Bourgoyen | 2013 |
| R-53520 | *Lactobacillus apis* | *B. terrestris* | Ledeganck | 2013 |
| R-53521 (LMG 28626) | *Bifidobacterium commune* | *B. hypnorum* | Wetteren | 2013 |
| R-53522 | *Lactobacillus bombicola* | *B. hypnorum* | Wetteren | 2013 |
| R-53523 | *Snodgrassella alvi* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53524 | *Snodgrassella alvi* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53525 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2013 |
| R-53526 | *Candida* sp. II | *B. pascuorum* | Gentbrugge | 2013 |
| R-53527 | *Lactobacillus bombicola* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53528 | *Snodgrassella alvi* | *B. hypnorum* | Wetteren | 2013 |
| R-53529 | *Commensalibacter intestini* | *B. hypnorum* | Wetteren | 2013 |
| R-53530 | *Commensalibacter intestini* | *B. hypnorum* | Wetteren | 2013 |
| R-53531 | *Snodgrassella alvi* | *B. hypnorum* | Wetteren | 2013 |
| R-53532 | *Lysinibacillus fusiformis* | *B. hypnorum* | Wetteren | 2013 |
| R-53533 | *Candida* sp. I | *B. hypnorum* | Wetteren | 2013 |
| R-53534 | *Fructobacillus tropaeoli* | *B. hypnorum* | Wetteren | 2013 |
| R-53535 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2013 |
| R-53536 | *Weissella viridescens* | *B. terrestris* | Ledeganck | 2013 |
| R-53537 | *Weissella bombi* | *B. terrestris* | Ledeganck | 2013 |
| R-53538 | *Weissella viridescens* | *B. terrestris* | Ledeganck | 2013 |
| R-53539 | *Lactobacillus bombicola* | *B. hypnorum* | Wetteren | 2013 |
| R-53540 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2013 |
| R-53541 | *Bifidobacterium actinocoloniiforme* | *B. terrestris* | Bourgoyen | 2013 |
| R-53542 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53553 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53554 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2013 |
| R-53555 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53556 | *Lactobacillus kimchicus* | *B. terrestris* | Bourgoyen | 2013 |
| R-53557 | *Lactococcus lactis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53558 | *Bombiscardovia coagulans* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53559 | *Bombiscardovia coagulans* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53560 | *Bifidobacterium commune* | *B. lapidarius* | Gentbrugge | 2013 |

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| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-53561 | *Lactobacillus bombicola* | *B. pascuorum* | Gentbrugge | 2013 |
| R-53583 (LMG 28360) | *Snodgrassella alvi* | *B. terrestris* | Ledeganck | 2013 |
| R-53630 | *Leuconostoc pseudomesenteroides* | *B. terrestris* | Bourgoyen | 2013 |
| R-53631 | *Hafnia alvei* | *B. terrestris* | Bourgoyen | 2013 |
| R-53632 | *Micrococcus luteus* | *B. pascuorum* | Bourgoyen | 2013 |
| R-53633 | *Snodgrassella alvi* | *B. pascuorum* | Bourgoyen | 2013 |
| R-53634 | *Snodgrassella alvi* | *B. pascuorum* | Ledeganck | 2013 |
| R-53635 | *Snodgrassella alvi* | *B. pascuorum* | Ledeganck | 2013 |
| R-53636 | *Hafnia alvei* | *B. terrestris* | Bourgoyen | 2013 |
| R-53637 | *Hafnia alvei* | *B. terrestris* | Bourgoyen | 2013 |
| R-53638 | *Lysinibacillus fusiformis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53639 | *Hafnia alvei* | *B. terrestris* | Bourgoyen | 2013 |
| R-53640 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2013 |
| R-53641 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2013 |
| R-53642 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2013 |
| R-53643 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2013 |
| R-53644 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2013 |
| R-53645 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2013 |
| R-53646 | *Lactobacillus kimchicus* | *B. terrestris* | Gentbrugge | 2013 |
| R-53647 | *Lysinibacillus fusiformis* | *B. lapidarius* | Coupure | 2013 |
| R-53648 | *Lysinibacillus fusiformis* | *B. pascuorum* | Coupure | 2013 |
| R-53649 | *Streptomyces albidoflavus* | *B. pascuorum* | Coupure | 2013 |
| R-53650 | *Lysinibacillus fusiformis* | *B. lapidarius* | Wetteren | 2013 |
| R-53651 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2013 |
| R-53652 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2013 |
| R-53653 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2013 |
| R-53654 | *Lactobacillus kunkeei* | *B. terrestris* | Bourgoyen | 2013 |
| R-53655 | *Candida bombi* | *B. pascuorum* | Coupure | 2013 |
| R-53656 | *Snodgrassella alvi* | *B. pascuorum* | Ledeganck | 2013 |
| R-53657 | *Moraxella osloensis* | *B. lapidarius* | Bourgoyen | 2013 |
| R-53658 | *Lactococcus lactis* | *B. terrestris* | Bourgoyen | 2013 |
| R-53659 | *Rosenbergiella nectarea* | *B. terrestris* | Bourgoyen | 2013 |
| R-53671 | *Candida bombi* | *B. lucorum* | Wetteren | 2014 |
| R-53672 | *Candida bombi* | *B. lucorum* | Wetteren | 2014 |
| R-53673 | *Hafnia alvei* | *B. pascuorum* | Bourgoyen | 2014 |
| R-53674 | *Candida* sp. I | *B. pascuorum* | Bourgoyen | 2014 |
| R-53675 | *Lysinibacillus fusiformis* | *B. lucorum* | Wetteren | 2014 |
| R-53676 | *Rosenbergiella nectarea* | *B. lucorum* | Wetteren | 2014 |
| R-53677 | *Snodgrassella alvi* | *B. lucorum* | Wetteren | 2014 |
| R-53678 | *Ureibacillus suwonensis* | *B. lapidarius* | Ledeganck | 2014 |
| R-53679 | *Paenibacillus barengoltzii* | *B. lapidarius* | Ledeganck | 2014 |
| R-53680 | *Snodgrassella alvi* | *B. lapidarius* | Ledeganck | 2014 |
| R-53681 | *Snodgrassella alvi* | *B. lapidarius* | Ledeganck | 2014 |
| R-53682 | *Candida bombiphila* | *B. lapidarius* | Ledeganck | 2014 |
| R-53683 | *Bacillus* sp. | *B. pascuorum* | Ledeganck | 2014 |
| R-53684 | *Candida bombiphila* | *B. pascuorum* | Ledeganck | 2014 |
| R-53685 | *Lactobacillus kunkeei* | *B. lucorum* | Wetteren | 2014 |
| R-53686 | *Serratia marcescens* | *B. lucorum* | Wetteren | 2014 |
| R-53687 | *Lactobacillus kunkeei* | *B. lucorum* | Wetteren | 2014 |
| R-53688 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |

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| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-53689 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2014 |
| R-53690 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2014 |
| R-53691 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2014 |
| R-53692 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2014 |
| R-53693 | *Lactobacillus kunkeei* | *B. terrestris* | Ledeganck | 2014 |
| R-53694 | *Fructobacillus fructosus* | *B. terrestris* | Ledeganck | 2014 |
| R-53695 | *Lactobacillus kunkeei* | *B. terrestris* | Ledeganck | 2014 |
| R-53696 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2014 |
| R-53697 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2014 |
| R-53698 | *Fructobacillus tropaeoli* | *B. lapidarius* | Gentbrugge | 2014 |
| R-53699 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2014 |
| R-53700 | *Lactobacillus bombicola* | *B. pascuorum* | Coupure | 2014 |
| R-53701 | *Lactococcus lactis* | *B. terrestris* | Bourgoyen | 2014 |
| R-53702 | *Lactobacillus bombicola* | *B. pascuorum* | Ledeganck | 2014 |
| R-53703 | *Hafnia alvei* | *B. lucorum* | Wetteren | 2014 |
| R-53704 | *Hafnia alvei* | *B. lucorum* | Wetteren | 2014 |
| R-53705 | *Gilliamella bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-53706 | *Leuconostoc mesenteroides* | *B. lapidarius* | Ledeganck | 2014 |
| R-53707 | *Leuconostoc mesenteroides* | *B. lapidarius* | Ledeganck | 2014 |
| R-53708 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-53709 | *Leuconostoc mesenteroides* | *B. lapidarius* | Ledeganck | 2014 |
| R-53710 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-53711 | *Bombiscardovia coagulans* | *B. lapidarius* | Ledeganck | 2014 |
| R-53713 | *Bacillus licheniformis* | *B. lapidarius* | Ledeganck | 2014 |
| R-53714 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-53715 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-53716 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-53717 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-53718 | *Fructobacillus tropaeoli* | *B. lucorum* | Wetteren | 2014 |
| R-53719 | *Lactococcus lactis* | *B. lucorum* | Wetteren | 2014 |
| R-53720 | *Lactobacillus bombicola* | *B. lucorum* | Wetteren | 2014 |
| R-53721 | *Bombiscardovia coagulans* | *B. lucorum* | Wetteren | 2014 |
| R-53722 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-53723 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-53724 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-53725 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-53726 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-53728 | *Lactobacillus bombicola* | *B. pascuorum* | Bourgoyen | 2014 |
| R-53729 | *Bombiscardovia coagulans* | *B. pascuorum* | Bourgoyen | 2014 |
| R-53730 | *Bombiscardovia coagulans* | *B. pascuorum* | Bourgoyen | 2014 |
| R-53731 | *Snodgrassella alvi* | *B. pascuorum* | Coupure | 2014 |
| R-53734 | *Lactobacillus kunkeei* | *B. terrestris* | Ledeganck | 2014 |
| R-53735 | *Fructobacillus tropaeoli* | *B. terrestris* | Gentbrugge | 2014 |
| R-53736 | *Hafnia alvei* | *B. lapidarius* | Bourgoyen | 2014 |
| R-53737 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-53738 | *Lactobacillus bombicola* | *B. pascuorum* | Ledeganck | 2014 |
| R-53740 | *Lactobacillus bombicola* | *B. pascuorum* | Ledeganck | 2014 |
| R-53741 | *Lactobacillus bombicola* | *B. pascuorum* | Bourgoyen | 2014 |
| R-54226 | *Enterococcus faecalis* | *B. terrestris* | Ledeganck | 2014 |

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| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-54227 | *Enterococcus faecalis* | *B. terrestris* | Ledeganck | 2014 |
| R-54228 | *Lactococcus garvieae* | *B. terrestris* | Ledeganck | 2014 |
| R-54229 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2014 |
| R-54230 | *Weissella bombi* | *B. terrestris* | Ledeganck | 2014 |
| R-54231 | *Lactococcus garvieae* | *B. terrestris* | Ledeganck | 2014 |
| R-54232 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-54233 | *Lactococcus lactis* | *B. lucorum* | Wetteren | 2014 |
| R-54234 | *Lysinibacillus fusiformis* | *B. lucorum* | Wetteren | 2014 |
| R-54235 | *Lactococcus lactis* | *B. lucorum* | Wetteren | 2014 |
| R-54236 | *Snodgrassella alvi* | *B. lucorum* | Wetteren | 2014 |
| R-54237 | *Lactobacillus bombicola* | *B. lucorum* | Wetteren | 2014 |
| R-54238 | *Lactobacillus kunkeei* | *B. lucorum* | Wetteren | 2014 |
| R-54239 | *Hafnia alvei* | *B. hypnorum* | Bourgoyen | 2014 |
| R-54240 (LMG 28624) | *Weissella bombi* | *B. hypnorum* | Bourgoyen | 2014 |
| R-54241 | *Weissella bombi* | *B. hypnorum* | Bourgoyen | 2014 |
| R-54242 | *Bombella intestini* | *B. lapidarius* | Coupure | 2014 |
| R-54243 | *Weissella bombi* | *B. terrestris* | Ledeganck | 2014 |
| R-54244 | *Weissella bombi* | *B. terrestris* | Ledeganck | 2014 |
| R-54245 | *Fructobacillus fructosus* | *B. terrestris* | Ledeganck | 2014 |
| R-54246 | *Lactobacillus kunkeei* | *B. lucorum* | Wetteren | 2014 |
| R-54247 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-54248 | *Lactobacillus kunkeei* | *B. lucorum* | Wetteren | 2014 |
| R-54249 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-54250 | *Convivina intestini* | *B. lucorum* | Wetteren | 2014 |
| R-54251 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-54252 | *Weissella bombi* | *B. hypnorum* | Bourgoyen | 2014 |
| R-54253 | *Weissella bombi* | *B. hypnorum* | Bourgoyen | 2014 |
| R-54382 | *Fructobacillus fructosus* | *B. lapidarius* | Coupure | 2014 |
| R-54642 | *Bombiscardovia coagulans* | *B. lapidarius* | Ledeganck | 2014 |
| R-54643 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-54644 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54645 | *Lactobacillus kunkeei* | *B. terrestris* | Ledeganck | 2014 |
| R-54646 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54647 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54648 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54649 | *Lactobacillus bombi* | *B. terrestris* | Ledeganck | 2014 |
| R-54650 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54651 | *Lactobacillus apis* | *B. terrestris* | Ledeganck | 2014 |
| R-54652 | *Bifidobacterium commune* | *B. terrestris* | Ledeganck | 2014 |
| R-54653 | *Lactobacillus kunkeei* | *B. terrestris* | Ledeganck | 2014 |
| R-54654 | *Fructobacillus fructosus* | *B. terrestris* | Ledeganck | 2014 |
| R-54655 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54656 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54657 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54658 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54659 | *Bifidobacterium commune* | *B. terrestris* | Ledeganck | 2014 |
| R-54660 | *Lactobacillus kunkeei* | *B. terrestris* | Ledeganck | 2014 |
| R-54661 | *Bifidobacterium bombi* | *B. terrestris* | Ledeganck | 2014 |
| R-54662 (LMG 29879$^{T}$) | *Gilliamella bombi* | *B. terrestris* | Ledeganck | 2014 |
| R-54663 | *Kocuria* sp. | *B. terrestris* | Ledeganck | 2014 |

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| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-54664 | *Fructobacillus fructosus* | *B. terrestris* | Ledeganck | 2014 |
| R-54665 | *Lactobacillus kunkeei* | *B. terrestris* | Gentbrugge | 2014 |
| R-54666 | *Enterobacter kobei* | *B. terrestris* | Gentbrugge | 2014 |
| R-54667 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2014 |
| R-54668 | *Vagococcus entomophilus* | *B. terrestris* | Gentbrugge | 2014 |
| R-54669 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2014 |
| R-54670 | *Lactobacillus kunkeei* | *B. terrestris* | Gentbrugge | 2014 |
| R-54671 | *Lactobacillus kunkeei* | *B. lucorum* | Wetteren | 2014 |
| R-54672 | *Bombiscardovia coagulans* | *B. lucorum* | Wetteren | 2014 |
| R-54673 | *Lactobacillus bombicola* | *B. lucorum* | Wetteren | 2014 |
| R-54674 | *Snodgrassella alvi* | *B. lucorum* | Wetteren | 2014 |
| R-54675 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-54676 | *Lactobacillus kunkeei* | *B. lucorum* | Wetteren | 2014 |
| R-54677 | *Fructobacillus fructosus* | *B. lucorum* | Wetteren | 2014 |
| R-54678 | *Snodgrassella alvi* | *B. lucorum* | Wetteren | 2014 |
| R-54679 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-54680 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-54681 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-54682 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-54684 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-54685 | *Lactococcus lactis* | *B. terrestris* | Gentbrugge | 2014 |
| R-54686 | *Lactobacillus bombi* | *B. terrestris* | Ledeganck | 2014 |
| R-54687 | *Lactobacillus kimchicus* | *B. terrestris* | Ledeganck | 2014 |
| R-54688 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-54689 | *Bifidobacterium bombi* | *B. lapidarius* | Ledeganck | 2014 |
| R-54690 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-54691 | *Lactobacillus bombicola* | *B. terrestris* | Ledeganck | 2014 |
| R-54692 | *Lactobacillus bombicola* | *B. pascuorum* | Ledeganck | 2014 |
| R-54693 | *Bombiscardovia coagulans* | *B. pascuorum* | Ledeganck | 2014 |
| R-54694 | *Lactobacillus bombicola* | *B. pascuorum* | Ledeganck | 2014 |
| R-54695 | *Snodgrassella alvi* | *B. terrestris* | Ledeganck | 2014 |
| R-54696 | *Lactobacillus bombicola* | *B. pascuorum* | Ledeganck | 2014 |
| R-54828 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-54829 | *Lactobacillus kunkeei* | *B. terrestris* | Coupure | 2014 |
| R-54830 | *Lactobacillus kunkeei* | *B. terrestris* | Coupure | 2014 |
| R-54831 | *Lactobacillus kunkeei* | *B. terrestris* | Coupure | 2014 |
| R-54832 | *Fructobacillus fructosus* | *B. terrestris* | Coupure | 2014 |
| R-54833 | *Lactobacillus bombicola* | *B. terrestris* | Coupure | 2014 |
| R-54834 | *Fructobacillus fructosus* | *B. terrestris* | Coupure | 2014 |
| R-54835 | *Lactobacillus kunkeei* | *B. terrestris* | Coupure | 2014 |
| R-54836 | *Lactobacillus kunkeei* | *B. terrestris* | Coupure | 2014 |
| R-54837 | *Fructobacillus tropaeoli* | *B. terrestris* | Coupure | 2014 |
| R-54838 | *Lactobacillus bombicola* | *B. terrestris* | Coupure | 2014 |
| R-54839 | *Fructobacillus fructosus* | *B. terrestris* | Coupure | 2014 |
| R-54840 | *Fructobacillus tropaeoli* | *B. terrestris* | Coupure | 2014 |
| R-54841 | *Snodgrassella alvi* | *B. terrestris* | Coupure | 2014 |
| R-54842 | *Fructobacillus fructosus* | *B. lapidarius* | Coupure | 2014 |
| R-54843 | *Fructobacillus fructosus* | *B. lapidarius* | Coupure | 2014 |
| R-54844 | *Fructobacillus tropaeoli* | *B. hypnorum* | Wetteren | 2014 |
| R-54845 | *Fructobacillus tropaeoli* | *B. hypnorum* | Wetteren | 2014 |

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| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-54846 | *Fructobacillus tropaeoli* | *B. hypnorum* | Wetteren | 2014 |
| R-54847 | *Fructobacillus tropaeoli* | *B. hypnorum* | Wetteren | 2014 |
| R-54848 | *Fructobacillus tropaeoli* | *B. hypnorum* | Wetteren | 2014 |
| R-54849 | *Lactobacillus bombicola* | *B. pascuorum* | Wetteren | 2014 |
| R-54850 | *Lactobacillus bombicola* | *B. pascuorum* | Wetteren | 2014 |
| R-54851 | *Lactobacillus bombicola* | *B. pascuorum* | Wetteren | 2014 |
| R-54852 (LMG 29880$^T$) | *Gilliamella mensalis* | *B. pascuorum* | Wetteren | 2014 |
| R-54853 | *Lactobacillus bombicola* | *B. terrestris* | Wetteren | 2014 |
| R-54854 | *Lactobacillus bombicola* | *B. terrestris* | Wetteren | 2014 |
| R-54855 | *Hafnia alvei* | *B. terrestris* | Wetteren | 2014 |
| R-54856 | *Serratia marcescens* | *B. terrestris* | Wetteren | 2014 |
| R-54857 | *Fructobacillus fructosus* | *B. terrestris* | Wetteren | 2014 |
| R-54858 | *Snodgrassella alvi* | *B. terrestris* | Wetteren | 2014 |
| R-54859 | *Lactobacillus bombicola* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54860 | *Fructobacillus tropaeoli* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54861 | *Fructobacillus fructosus* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54862 | *Fructobacillus fructosus* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54863 | *Snodgrassella alvi* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54864 | *Fructobacillus tropaeoli* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54865 | *Lactobacillus bombicola* | *B. lapidarius* | Ledeganck | 2014 |
| R-54866 | *Fructobacillus fructosus* | *B. terrestris* | Coupure | 2014 |
| R-54867 | *Lactobacillus apis* | *B. terrestris* | Coupure | 2014 |
| R-54868 | *Fructobacillus fructosus* | *B. terrestris* | Coupure | 2014 |
| R-54869 | *Bombiscardovia coagulans* | *B. terrestris* | Coupure | 2014 |
| R-54870 | *Lactobacillus bombicola* | *B. terrestris* | Coupure | 2014 |
| R-54871 | *Lactobacillus bombicola* | *B. terrestris* | Coupure | 2014 |
| R-54872 | *Fructobacillus tropaeoli* | *B. terrestris* | Coupure | 2014 |
| R-54873 | *Fructobacillus fructosus* | *B. terrestris* | Coupure | 2014 |
| R-54874 | *Leuconostoc pseudomesenteroides* | *B. terrestris* | Coupure | 2014 |
| R-54875 | *Leuconostoc pseudomesenteroides* | *B. terrestris* | Coupure | 2014 |
| R-54876 | *Leuconostoc* sp. | *B. lapidarius* | Coupure | 2014 |
| R-54877 | *Fructobacillus tropaeoli* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54878 | *Fructobacillus tropaeoli* | *B. lapidarius* | Bourgoyen | 2014 |
| R-54879 | *Fructobacillus tropaeoli* | *B. lapidarius* | Bourgoyen | 2014 |
| R-55198 | *Candida bombi* | *B. lapidarius* | Wetteren | 2015 |
| R-55199 | *Lactococcus lactis* | *B. lapidarius* | Wetteren | 2015 |
| R-55200 | *Bombella intestini* | *B. lapidarius* | Wetteren | 2015 |
| R-55201 | *Candida bombi* | *B. lapidarius* | Wetteren | 2015 |
| R-55202 | *Bombiscardovia coagulans* | *B. lapidarius* | Wetteren | 2015 |
| R-55203 | *Fructobacillus tropaeoli* | *B. lapidarius* | Wetteren | 2015 |
| R-55204 | *Fructobacillus tropaeoli* | *B. lapidarius* | Wetteren | 2015 |
| R-55206 | *Candida bombi* | *B. hypnorum* | Bourgoyen | 2015 |
| R-55207 | *Candida bombi* | *B. hypnorum* | Bourgoyen | 2015 |
| R-55208 | *Lactobacillus* sp. | *B. hypnorum* | Bourgoyen | 2015 |
| R-55209 | *Candida* sp. I | *B. hypnorum* | Bourgoyen | 2015 |
| R-55210 | *Fructobacillus fructosus* | *B. hypnorum* | Bourgoyen | 2015 |
| R-55211 | *Fructobacillus tropaeoli* | *B. terrestris* | Bourgoyen | 2015 |
| R-55212 | *Fructobacillus fructosus* | *B. terrestris* | Bourgoyen | 2015 |
| R-55213 | *Bifidobacterium commune* | *B. terrestris* | Bourgoyen | 2015 |
| R-55214 | *Fructobacillus tropaeoli* | *B. terrestris* | Bourgoyen | 2015 |

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| Isolate (LMG number) | Identification | Bumblebee species | Sampling location | Isolation year |
|---|---|---|---|---|
| R-55215 | *Snodgrassella alvi* | *B. terrestris* | Bourgoyen | 2015 |
| R-55216 | *Lactobacillus kunkeei* | *B. terrestris* | Bourgoyen | 2015 |
| R-55217 | *Snodgrassella alvi* | *B. terrestris* | Bourgoyen | 2015 |
| R-55218 | *Lactobacillus kunkeei* | *B. terrestris* | Bourgoyen | 2015 |
| R-55219 | *Fructobacillus tropaeoli* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55220 | *Fructobacillus tropaeoli* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55221 | *Lactobacillus bombicola* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55222 | *Lactobacillus bombicola* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55223 | *Snodgrassella alvi* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55224 | *Bombiscardovia coagulans* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55225 | *Fructobacillus fructosus* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55226 | *Fructobacillus fructosus* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55227 | *Fructobacillus tropaeoli* | *B. pascuorum* | Bourgoyen | 2015 |
| R-55228 | *Lactobacillus kunkeei* | *B. terrestris* | Bourgoyen | 2015 |
| R-55229 | *Fructobacillus tropaeoli* | *B. terrestris* | Bourgoyen | 2015 |
| R-55230 | *Enterococcus faecalis* | *B. terrestris* | Bourgoyen | 2015 |
| R-55231 | *Fructobacillus fructosus* | *B. terrestris* | Bourgoyen | 2015 |
| R-55232 | *Candida bombi* | *B. terrestris* | Bourgoyen | 2015 |
| R-55233 | *Fructobacillus fructosus* | *B. terrestris* | Bourgoyen | 2015 |
| R-55234 | *Fructobacillus tropaeoli* | *B. terrestris* | Bourgoyen | 2015 |
| R-55235 | *Fructobacillus tropaeoli* | *B. terrestris* | Bourgoyen | 2015 |
| R-55236 | *Candida bombi* | *B. terrestris* | Bourgoyen | 2015 |
| R-55237 | *Fructobacillus tropaeoli* | *B. terrestris* | Bourgoyen | 2015 |
| R-55238 | *Hafnia alvei* | *B. terrestris* | Bourgoyen | 2015 |
| R-55239 | *Lactobacillus bombicola* | *B. terrestris* | Bourgoyen | 2015 |
| R-55240 | *Fructobacillus tropaeoli* | *B. terrestris* | Bourgoyen | 2015 |
| R-55241 | *Candida bombi* | *B. lapidarius* | Wetteren | 2015 |
| R-55242 | *Lactobacillus bombicola* | *B. lapidarius* | Wetteren | 2015 |
| R-55243 | *Snodgrassella alvi* | *B. lapidarius* | Wetteren | 2015 |
| R-55244 | *Lactobacillus kunkeei* | *B. lapidarius* | Wetteren | 2015 |
| R-55245 | *Candida bombi* | *B. lapidarius* | Wetteren | 2015 |
| R-55246 | *Candida* sp. I | *B. pascuorum* | Wetteren | 2015 |
| R-55247 | *Candida bombi* | *B. pascuorum* | Wetteren | 2015 |
| R-55248 | *Lactobacillus bombicola* | *B. pascuorum* | Wetteren | 2015 |
| R-55249 | *Snodgrassella alvi* | *B. pascuorum* | Wetteren | 2015 |
| R-55250 | *Fructobacillus tropaeoli* | *B. pascuorum* | Wetteren | 2015 |
| R-55251 | *Bombiscardovia coagulans* | *B. pascuorum* | Wetteren | 2015 |
| R-55252 | *Lactobacillus bombicola* | *B. pascuorum* | Wetteren | 2015 |
| R-55253 | *Lysinibacillus fusiformis* | *B. pascuorum* | Wetteren | 2015 |
| R-55254 | *Snodgrassella alvi* | *B. pascuorum* | Wetteren | 2015 |
| R-55255 | *Lactobacillus bombicola* | *B. pascuorum* | Wetteren | 2015 |
| R-55256 | *Snodgrassella alvi* | *B. pascuorum* | Wetteren | 2015 |
| R-55257 | *Bombiscardovia coagulans* | *B. pascuorum* | Wetteren | 2015 |
| R-55258 | *Zygosaccharomyces rouxii* | *B. lapidarius* | Wetteren | 2015 |
| R-55278 | *Bombiscardovia coagulans* | *B. terrestris* | Bourgoyen | 2015 |
| R-55279 | *Bombiscardovia coagulans* | *B. lapidarius* | Wetteren | 2015 |
| R-55280 | *Lactobacillus kunkeei* | *B. terrestris* | Bourgoyen | 2015 |
| R-55281 | *Kocuria rhizophila* | *B. hypnorum* | Bourgoyen | 2015 |

# Part VII

# Acknowledgements

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