Ghent University
Faculty of Sciences
Department of Biochemistry and Microbiology

New insights into the isolation, identification and taxonomy of acetic acid bacteria through the application of matrix assisted laser desorption/ionization time-of-flight mass spectrometry and whole genome sequence analysis

Leilei Li

Promoter: Prof. Dr. Peter Vandamme
Supervisor: Dr. Ilse Cleenwerck

Dissertation submitted in partial fulfillment of the requirements
for the degree of Doctor of Biochemistry and Biotechnology.

Examination committee

Prof. Dr. Savvas Savvides (Chairman)
L-Probe: Laboratory for Protein Biochemistry and Biomolecular Engineering, Faculty of Sciences, Ghent University, Ghent, Belgium

Prof. Dr. Peter Vandamme (Promoter)
LM-UGent: Laboratory of Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium

Dr. Ilse Cleenwerck (Supervisor)
BCCM/LMG Bacteria Collection, Faculty of Sciences, Ghent University, Belgium

Dr. Francis. Gosselé
Phibro Animal Health, Pfizer, SmithKline Beecham Biological, The United States of America

Prof. Dr. François Barja
Microbiology Unit, Botany and Plant Biology Dept., University of Geneva, Switzerland

Prof. Dr. Guy Smagghe
Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University, Belgium

Prof. Dr. Jean Swings
LM-UGent: Laboratory of Microbiology, Faculty of Sciences, Ghent University, Ghent, Belgium

Prof. Dr. Stefan Weckx
Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Brussels, Belgium

Acknowledgements

I knew it wouldn't be easy to leave everyone and everything I love 8000 kilometers behind for four years, and it was not. Still, it's been a pleasure and honor to do a Ph.D in one of the best microbiology labs in the world, to live in Ghent, to make new friends and form my own family. For that, I would like to express my sincere appreciation to everybody who has been there for me in the past four years.

First and foremost I want to thank my promoter Prof. Dr. Peter Vandamme. He provided me a chance and led me into the field of bacterial taxonomy. I appreciate all his contributions of time, ideas and energy to make my Ph.D experience productive and stimulating. I'm especially grateful to my supervisor Dr. Ilse Cleenwerck for guiding me step by step both in practical work and writing. She selflessly shared her knowledge and experience in work, which has been of great value to me.

A big part of the work presented in this dissertation is the result of collaboration and help from Prof. Dr. Luc De Vuyst and all coworkers from his lab. Especially Koen, Simon, Tom, Wim and Prof. Dr. Stefan Weckx, who have contributed their own time to help me to conduct experimental work.

Many thanks to our "MALDIers", Anneleen, Freek and Maarten for all the theoretical and hand-on sessions on MALDI-TOF MS and collaboration on publications. Thanks to Isabel, Charlotte, Jessy and Eliza for sharing their knowledge, organizing group activities and having fun together. I want to thank Anne, Jindrich, Timo, Joanna and Leentje, especially in the last period of my Ph.D, when they were always there, willing to hear me out when I need to talk and give me energy to keep going. Not to mention Marjan and Katrien, my first and last year lab mates, who have given me help on more than work related matters and I wish them all the best. Lots of thanks to Margo, Cindy and Evie, for

all their help on lab work, for all the care they gave me and for all the vegetables and eggs from Margo's garden. I want to thank Kim, Helen and Bram for the very interesting time in South Korea in 2014. I'd like to thank Yihua, to whom I can always talk in Chinese and I wish her all the best finishing her Ph.D. I also want to give my sincere thanks to Jeanine, Bart and Liesbeth for providing such a proper and organized lab environment. Thank Pia, Liesje, Els and all the colleagues for all the kindness they have given to me.

A special thanks goes to Diya and Qianqian, they are not only my friends but also my family. To be friends with them is one of the best things I've ever done in my life. No matter where we will be in the future, I will always think of them. Wish them all the best in their Ph.D, in future jobs and in love life! Thank my Belgian friends, Willem, Mechtild, Lieven, Jannick and Ilse, for traveling so far to China and witness my wedding, for all the fun we had and will still have going snowboarding every year! Thank my Chinese-speaking Belgian friends Leen, Vincent and Ann for all the language practicing evening and delicious food!

I'd thank my families, mama Rita, papa Paul, uncle Luc, aunt Katrien, Cindy, Ilse, Tom and Lien. Thank you all for accepting me in this family and make me feel at home. Thank my dearest aunt Lifang and uncle Chunlei for taking me as their own daughter. Without their wise advice and support, I wouldn't have gone so far.

Last but not least, I'd give my sincere thanks to my love Wim De Smet. Thanks for showing up in the coffee room, for loving me, marrying me, supporting me whenever I need it. You completed my life!

感谢此刻远在中国的所有关心爱护我的家人和朋友。感谢爸爸妈妈！女儿今天所有的一切都离不开爸妈三十年来辛勤的养育。这本博士毕业论文和博士学位是女儿的也是爸爸妈妈的。

愿用寸草心，来报三春晖。我永远爱你们！

Gent, September 17, 2015
Leilei Li

Contents

Examination committee i

Acknowledgements iii

List of Figures ix

List of Tables xiii

Acronyms xv

I. Introduction 1

Background and objectives 3

Outline 5

II. Literature Overview 7

1. Acetic acid bacteria (AAB) 9
   1.1. General introduction on AAB 9
      1.1.1. Ecological niches 10
      1.1.2. Metabolites of AAB 16
      1.1.3. Applications of AAB 18
   1.2. AAB as insect symbionts 23
   1.3. Genome studies of AAB 27

2. AAB taxonomy **35**
2.1. History of AAB taxonomy 35
2.2. Polyphasic taxonomy 38
2.2.1. Phenotypic analysis 38
2.2.2. Chemotaxonomic analysis 39
2.2.3. Genotypic analysis 40
2.3. Identification of AAB using MALDI-TOF MS 45
2.3.1. Overview of MALDI-TOF MS 45
2.3.2. MALDI-TOF MS in microbiology 46
2.3.3. Application of MALDI-TOF MS for AAB identification 48
2.4. Genomic taxonomy 49

III. Experimental Work **53**

3. Improved classification of acetic acid bacteria through MALDI-TOF MS: *Gluconobacter nephelii* and *Gluconobacter uchimurae* are the later heterotypic synonyms of *Gluconobacter japonicus* and *Gluconobacter oxydans*, respectively **57**
3.1. Introduction 59
3.2. Material and methods 60
3.2.1. Strains 60
3.2.2. MALDI-TOF MS analysis 61
3.2.3. 16S rRNA gene sequence analysis, AFLP, MLSA and DNA-DNA hybridizations 62
3.2.4. Whole-genome sequencing (WGS), assembly and calculation of ANI values 63
3.2.5. Phenotypic tests 63
3.3. Results 63
3.3.1. Medium selection 63
3.3.2. The MALDI-TOF MS AAB identification database 64
3.3.3. Identification of cocoa bean isolates 68
3.3.4. Identification of the BCCM/LMG Bacteria Collection strains 69

3.3.5. MLSA analysis . . . 71
3.3.6. Whole-genome sequencing and ANI calculation . 71
3.3.7. Phenotypic analysis of *G. japonicus*/*G. nephelii* and *G. oxydans*/*G. uchimurae* strains . . . 73
3.4. Discussion . . . 73
3.5. Acknowledgements . . . 78
3.6. Supplementary materials . . . 78

4. *Acetobacter sicerae* sp. nov., isolated form cider and kefir and identification of *Acetobacter* species by *dnaK*, *groEL* and *rpoB* sequence analysis 99
4.1. Introduction . . . 101
4.2. Methods, results and discussion . . . 101
4.3. Conclusion . . . 112
4.4. Acknowledgements . . . 112
4.5. Supplementary materials . . . 114

5. *Bombella intestini* gen. nov., sp. nov., an acetic acid bacterium isolated from bumble bee crop 123
5.1. Introduction . . . 125
5.2. methods, results and discussion . . . 125
5.3. Conclusion . . . 132
5.4. Acknowledgements . . . 134
5.5. Supplementary materials . . . 134

6. Whole genome sequence analysis of *Bombella intestini* LMG 28161^{T}, a novel acetic acid bacterium isolated from the crop of a red-tailed bumble bee, *Bombus lapidarius* 141
6.1. Introduction . . . 142
6.2. Materials and methods . . . 144
6.3. Results and discussion . . . 146
6.4. Conclusion . . . 156
6.5. Acknowledgements . . . 156

IV. General Discussion and Future Perspectives 157

7. MALDI-TOF MS: a rapid, high-throughput approach for classification and identification of AAB 161
7.1. Call for a quick identification method for acetic acid bacteria . . . 161
7.2. MALDI-TOF MS for AAB identification and classification 162
7.2.1. Instrument and software requirements,cultivation conditions, sample preparation . . . 162
7.2.2. Taxonomic resolution of MALDI-TOF MS . . . 164
7.3. MALDI-TOF MS allows to discover novel AAB . . . 169
7.4. Isolation strategies for AAB and MALDI-TOF MS for the dereplication of AAB isolates . . . 173

8. Defining AAB in the genomic era 175
8.1. The current practice of polyphasic taxonomy of AAB . . 176
8.2. Genomic approach towards AAB classification . . . 178

V. Summary 183

Nederlandstalige samenvatting 185

English summary 187

VI. Bibliography 193

VII. Annex 233

List of Figures

2.1. ML tree based on 16S rRNA sequences of the type strains of all species of family *Acetobacteraceae*. 42

3.1. MALDI-TOF MS dendrogram of AAB reference strains. 65

3.2. ML tree based on concatenated sequences of 3 house-keeping gene fragments of the genus *Gluconobacter*. . . . 72

S3.1. MALDI-TOF MS profiles of *Komagataeibacter europaeus* strains. 79

S3.2. UPGMA dendrogram based on MALDI-TOF MS profiles of *Acetobacter pasteurianus* LMG 1604, LMG 1587 and LMG 1552, *Acetobacter* sp. R-49137, strains of *Acetobacter cerevisiae* and the type strain of *Acetobacter malorum*. 80

S3.3. AFLP™ DNA fingerprints of (A) *G. japonicus* and *G. nephelii* strains and their closest phylogenetic relatives; (B) *G. oxydans*, *G. uchimurae* and their closest phylogenetic ralative; (C). *A. senegalensis* and *A. tropicalis* strains. 81

S3.4. ML tree based on 16S rRNA gene sequences of the type strains of all species of family *Acetobacteraceae*. 82

4.1. MALDI-TOF MS profiles of *Acetobacter sicerae* sp. nov. and their closest phylogenetic relative, *Acetobacter aceti*. . . 102

4.2. ML tree based on 16S rRNA gene sequences showing the pylogenetic position of *Acetobacter sicerae* sp. nov. within the genus *Acetobacter*. 104

4.3. ML tree based on concatenated sequences of 3 housekeeping gene fragments showing the phylogenetic position of *Acetobacter sicerae* sp. nov. within the genus *Acetobacter*. 106
4.4. AFLP™ fingerprints of *Acetobacter sicerae* sp. nov. and their closest phylogenetic relatives. 107
4.5. RAPD fingerprints of *Acetobacter sicerae* sp. nov. strains. 108
S4.1. Comparison of MALDI-TOF MS profiles of *Acetobacter sicerae* strains. 114
S4.2. ML tree based on concatenated amino acids sequences of 3 housekeeping gene fragments of *Acetobacter* species. . 115
S4.3. ML tree of all established *Acetobacter* species based on partial *groEL* gene sequences. 116
S4.4. ML tree of all established *Acetobacter* species based on partial *groEL* gene amino acid sequences. 117

5.1. ML tree based on 16S rRNA gene sequences showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. within the family *Acetobacteraceae*. 128
S5.1. MALDI-TOF MS identification of bumble bee isolates as *Asaia astilbis* and "*Commensalibacter intestini*". 137
S5.2. 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}. 137
S5.3. NJ tree based on 16S-23S rRNA ITS sequences showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. to its close relatives. 138
S5.4. ML tree based on partial housekeeping gene sequences *groEL*, showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. to its close relatives. 138
S5.5. ML tree based on amino acid sequences of housekeeping gene *groEL* fragments, showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. to its close relatives. 139
S5.6. RAPD fingerprints of *Bombella intestini* gen. nov., sp. nov. strains. 139

6.1. Central metabolic pathways of *Bombella intestini* LMG 28161^{T}. 150
6.2. Membrane bound dehydrogenase and respiratory chain of *Bombella intestini* LMG 28161^{T}. 152
6.3. Growth and D-glucose consumption by *Bombella intestini* LMG 28161^{T} during fermentation in basal medium with 1 % (w/v) D-glucose. 154
6.4. Carbohydrate consumption by *Bombella intestini* LMG 28161^{T} under aerobic and micro-aerobic conditions. . . . 155

7.1. MALDI-TOF MS dendrogram showing strain delineated clusters. 167
7.2. MALDI-TOF MS profiles of *Gluconacetobacter sacchari* strains. 168
7.3. ML tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strains within the genus *Bombella*. 171

List of Tables

1.1. Currently established AAB genera and species and the isolation source of their type strain. 11
1.2. AAB and related insects hosts. 25
1.3. List of AAB strains with publicly available genome sequences at the time of writing. 31

3.1. List of isolates of cocoa bean fermentation. MALDI-TOF MS identifications. 70
3.2. Average Nucleotide Identity values among draft whole genome sequences of selected AAB strains. 74
S3.1. List of AAB strains selected for the construction of a MALDI-TOF MS identification database. 83
S3.2. List of the poorly characterized BCCM/LMG strains selected for identification through MALDI-TOF MS. . . 88
S3.3. Results of reciprocal DNA-DNA hybridizations. 93
S3.4. Accession numbers of housekeeping genes sequences obtained in this study. 94
S3.5. General features of genomes sequenced in this study. . . 95
S3.6. Phenotypic features of strains of *G. japonicus* and *G. nephelii*. 96
S3.7. Phenotypic features of strains of *G. uchimurae* and *G. oxydans*. 97

4.1. Cellular fatty acid contents of *Acetobacter sicerae* sp. nov. and all *Acetobacter* type strains. 110

4.2. Differential characteristics between *Acetobacter sicerae* sp. nov. (LMG 1531^{T} and LMG 27543) and the phylogenetic closest *Acetobacter* species. 111
S4.1. Accession numbers of housekeeping genes sequences of *dnaK*, *groEL* and *rpoB*. 118
S4.1. Accession numbers of housekeeping genes sequences of *dnaK*, *groEL* and *rpoB*. 119
S4.2. DNA-DNA relatedness of *A. sicerae* and the type strains of its closest phylogenetic relatives. 120
S4.3. Characteristics between *Acetobacter sicerae* and all established *Acetobacter* species. 120
S4.3. Differential characteristics between *Acetobacter sicerae* and all established *Acetobacter* species. 121

5.1. Cellular fatty acid contents of *Bombella intestini* gen. nov., sp. nov. type strain and related type strains. . . . 130
5.2. Characteristics that differentiate *Bombella* gen. nov. from its phylogenetic close relatives of the family *Acetobacteraceae*. 133
S5.1. Isolates from bumble bee gut samples and their identifications. 135
S5.2. Cellular fatty acid contents of *Bombella intestini* gen. nov., sp. nov. and type strains of all the type species of the family *Acetobacteraceae*. 136

7.1. List of novel AAB discovered by MALDI-TOF MS and confirmed by 16S rRNA or housekeeping gene sequence analysis. 172

Acronyms

A.	*Acetobacter*
As.	*Asaia*
AAB	acetic acid bacteria
AAI	average amino acid identity
AAM	acetic acid medium
AFLP	amplified fragment length polymorphism
ANI	average nucleotide identity
ATCC	American Type Culture Collection, Manassas, VA, USA
ATP	adenosine triphosphate
B.	*Bombella*
BCC	BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology (BIOTEC), Khlong Luang, Pathumthani, Thailand
BCCM	Belgian Coordinated Collections of Microorganisms
BLAST	basic local alignment tool
CDs	coding sequence
CGD	chronic granulomatous disease
COX	cytochrome oxidase
CRISPRs	clustered regularly interspaced short palindromic repeats
dnaK	heat shock protein encoding gene

DDH	DNA-DNA hybridization
DNA	deoxyribonucleic acid
DSM	DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
EMP	Embden-Meyerhof-Parnas pathway
FAD(H)	flavin adenine dinucleotide
Ga.	*Gluconacetobacter*
G.	*Gluconobacter*
groEL	molecular chaperone *groEL* gene
GGDC	genome-to-genome distance calculator
GTP	guanosine triphosphate
HGT	horizontal gene transfer
IFO	Institute for Fermentation, Osaka, Yodogawa-ku, Osaka, Japan
ITS	internal transcribed spacer
JCM	Japan Collection of Microorganisms, RIKEN BioResource Center, Japan
Kom.	*Komagataeibacter*
KEGG	kyoto encyclopedia of genes and genomes
LGT	lateral gene transfer
LMG	laboratory of microbiology, Ghent University
MALDI	matrix assisted laser desorption/ionization
MEGA	molecular evolutionary genetics analysis
ML	maximum-likelihood
MLSA	multilocus sequence analysis

MS mass spectrometry

NADP(H) nicotinamide adenine dinucleotide phosphate
NBRC NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan
NCBI National center for biotechnology information
NGS next-generation sequencing
NJ neighbour joining

ORF open reading frame

PCR polymerase chain reaction
PGAAP prokaryotic genome automatic annotation pipeline
POCP percentage of conserved proteins
PPP pentose-phosphate pathway
PQQ cofacter pyrroloquinoline quinone

Q-10 coenzyme ubiquinone-10
Q-9 colenzyme ubiquinone-9

rpoB β-subunit of the bacterial RNA polymerase
RAPD random amplification of polymorphic
RAST rapid annotation using subsystems technology
rep-PCR repetitive element sequence-based PCR
RFLP restriction fragment length polymorphisms analysis
RNA ribonucleic acid
rRNA ribosomal RNA

S. *Saccharibacter*

T type strain
TCA tricarboxylic acid cycle
TOF time-of-flight

UOX ubiquinone oxidase
UPGMA unweighted pair group method with arithmetic means

WGS whole genome sequence

Part I.

Introduction

Background and objectives

Acetic acid bacteria (AAB) are a group of food related and industrial microorganisms. They can be both beneficial and detrimental to food or beverage fermentation. Some AAB species are opportunistic human pathogens, while some others evolved symbiotic relationships with various insects. Given their relevance in industry, medicine and agriculture, their isolation and quick identification are required to obtain a better understanding of these organisms.

Many AAB have fastidious growth characteristics which render them difficult to isolate. Various enrichment and selective media have been proposed for the isolation of AAB from different sources. Most of the AAB selective media have a pH below 4.5 and contain a variety of sugar and/or sugar alcohols as carbon source. A combination of different isolation procedures is often recommended to isolate as many AAB as possible. The current classification of AAB is a result of polyphasic taxonomic studies, which combine phenotypic, genotypic and chemotaxonomic characterization methods in order to provide a comprehensive species description. These polyphasic studies also yielded an array of identification methods. However, the labor intensive, expensive and inaccurate nature of (some of) these routine identification techniques underscore the need for more rapid, more cost-effective and accurate methods for AAB identification. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been proven useful in categorization and identification of various microbes, especially in routine identification of clinical microorganisms. The accuracy of bacterial identification using MALDI-TOF MS largely depends on the robustness of the reference database, and therefore, the construction and validation of such a reference database is crucial.

In the meantime, with the rapid development of sequencing technology, it is feasible to obtain the whole genome sequence (WGS) of any bacterial strain through next-generation sequencing (NGS). Efforts are being made to integrate genomic information into microbial taxonomy and systematics, which will also greatly address the current taxonomy pitfalls. Beyond its taxonomic value, genomic information allows a better understanding of microbial evolution and function in the environment, especially when combined with laboratory experiments.

The goals of the present study are **(i)** to develop and evaluate a robust MALDI-TOF MS database for the identification of novel AAB isolates; **(ii)** to improve the current AAB classification by means of whole-genome sequence based approaches, and **(iii)** to explore the genetic nature of a novel AAB species described in the course of the present study, *i.e.* *Bombella intestini* through whole-genome sequence analysis of the type strain of this species, LMG 28161^{T}.

Outline

Part II consists of a comprehensive **literature overview** of AAB and their taxonomy. **Chapter 1** describes their ecological niches, unique metabolites, applications in food and beverage fermentations and industry, and their recently revealed role as insect endosymbionts. It concludes with an overview of recent whole genome sequence based studies of AAB. **Chapter 2** reviews the history and recent developments in AAB taxonomy, illustrates the usefulness of MALDI-TOF MS for the fast and high-throughput identification of AAB, as well as the importance of genome sequence studies for AAB taxonomy.

Part III presents a series of **experimental studies** performed in the frame of the present Ph.D study. **Chapter 3** describes the construction and evaluation of a MALDI-TOF MS identification database for AAB, along with several taxonomic rearrangements that resulted from these studies. Subsequently, isolates from various sources were investigated and subjected to identification using this database. We applied novel isolation strategies and multiple selective media for the isolation of AAB from bumble bee gut samples. Novel AAB species including *Acetobacter sicerae* sp. nov. (**Chapter 4**) and *Bombella intestini* gen. nov., sp. nov. (**Chapter 5**) were discovered during these studies and were formally described using multilocus sequence analysis (MLSA) and a polyphasic taxonomic approach. Finally, we determined the whole genome sequence of the *Bombella intestini* type strain in order to explore its genetic nature (**Chapter 6**).

Part IV comprises a **general discussion** of the results and some **future perspectives**.

Part II.

Literature Overview

1 Acetic acid bacteria (AAB)

1.1. General introduction on AAB

The acetic acid bacteria (AAB) are an acetous group of bacteria and are classified in the family *Acetobacteraceae*. Their cells are Gram-stain-negative, rod-shaped or coccoid, and peritrichously or polarly flagellated when motile. Catalase activity is generally present (but absent in *Acetobacter peroxydans*); oxidase activity is absent. They are able to oxidize a wide range of sugars, alcohols and sugar alcohols to corresponding acids. At present, AAB are classified into 18 genera and 85 species. Over the years, AAB have attracted much attention due to their metabolite traits that are of industrial interest. One of their best known characteristics is the production of vinegar. The earliest studies of AAB were done on vinegar, and historically AAB were referred to as "vinegar bacteria". Nowadays, the context of AAB has expanded to not only include vinegar related AAB, but also other bacteria that interact with flowers, fruits, plants, insects and even human.

1.1.1. Ecological niches

AAB are widespread in natural and human environments. In the natural environment, they are often found in fruits, flowers and insects. In warm and humid climates such as in Thailand and Sri Lanka, they are commonly associated with different types of flowers, fruits and rhizosphere soil (Gosselé and Swings, 1986; Lisdiyanti *et al.*, 2000; Van Keer *et al.*, 1981; Yukphan *et al.*, 2004b). Many of them have established a symbiotic relationship with insects (Crotti *et al.*, 2010), and some are considered opportunistic human pathogens (Greenberg *et al.*, 2006). In manmade environments, they are widely distributed in fermented foods and beverages, such as vinegar, wine, beer, cider and kombucha (Carr, 1958; Dutta and Gachhui, 2006; Gosselé *et al.*, 1984; Spitaels *et al.*, 2014a). Interestingly, some of these above-mentioned niches are anaerobic or micro-aerobic, meaning that AAB are not strict aerobes as they were traditionally considered. An overview of AAB species and the isolation source of the corresponding type strains is listed in Table 1.1.

Table 1.1 illustrates that *Acetobacter* species are often isolated from alcoholic niches, such as beer or wine, but also from fruit and vinegar. *Gluconacetobacter* species are often recovered from environmental niches, such as plain soil or rhizosphere soil. *Komagataeibacter* species are mostly isolated from vinegar fermentation, which is highly acidic. *Gluconobacter* species are generally tolerant to a high sugar concentration, and are mainly isolated from sugar-rich niches, such as fruits and flowers. *Asaia* species are most frequently found in tropical flowers. *Bombella* and *"Commensalibacter"* species are originally isolated from insects. It is noteworthy that besides *Bombella* and *"Commensalibacter"*, other AAB such as *Acetobacter*, *Asaia*, *Gluconobacter*, *Gluconacetobacter* and *Saccharibacter* are all found associated with different insects (Crotti *et al.*, 2010). Although only *Granulibacter bethesdensis* was originally isolated from a patient with the chronic granulomatous disease, some other species, such as *As. bogorensis*, *As. lannensis*, *A. cibinogensis*, *A. indonesiensis*, *Gluconobacter.* spp and *Acidomonas methanolic* were all found in human patients (Komagata *et al.*, 2014).

Table 1.1. Currently established AAB genera and species and the isolation source of their type strain.

Genus	Species	Isolation source	Reference
ACETOBACTER			
Acetobacter	*aceti*	Beechwood shavings of a vinegar plant	(Beijerinck, 1898)(Buchanan *et al.*, 1966)
Acetobacter	*cerevisiae*	Beer (ale)	(Cleenwerck *et al.*, 2002)
Acetobacter	*cibinongensis*	Fruit	(Lisdiyanti *et al.*, 2001)
Acetobacter	*estunensis*	Cider	(Carr, 1958)(Lisdiyanti *et al.*, 2000)
Acetobacter	*fabarum*	Cocoa heap fermentation	(Cleenwerck *et al.*, 2008)
Acetobacter	*farinalis*	Fermented rice flour	(Tanasupawat *et al.*, 2011b)
Acetobacter	*ghanensis*	Cocoa bean heap fermentation	(Cleenwerck *et al.*, 2007)
Acetobacter	*indonesiensis*	Fruit of zirzak	(Lisdiyanti *et al.*, 2000)
Acetobacter	*lovaniensis*	Sewage on soil	(Lisdiyanti *et al.*, 2000)
Acetobacter	*malorum*	Rotten apple	(Cleenwerck *et al.*, 2002)
Acetobacter	*nitrogenifigens*	Kombucha tea	(Dutta and Gachhui, 2006)
Acetobacter	*oeni*	Spoiled red wine	(Silva *et al.*, 2006)
Acetobacter	*okinawensis*	Sugarcane	(Iino *et al.*, 2012a)
Acetobacter	*orientalis*	Canna flower	(Lisdiyanti *et al.*, 2000)
Acetobacter	*orleanensis*	Beer	(Henneberg, 1906; Lisdiyanti *et al.*, 2000)
Acetobacter	*papayae*	Papaya fruit	(Iino *et al.*, 2012a)
Acetobacter	*pasteurianus*	Beer	(Lisdiyanti *et al.*, 2000)
Acetobacter	*peroxydans*	Ditchwater	(Gullo and Giudici, 2009)
Acetobacter	*persici*	Peach fruit	(Iino *et al.*, 2012a)
Acetobacter	*pomorum*	Cider vinegar fermentation	(Sokollek *et al.*, 1998)
Acetobacter	*senegalensis*	Mango fruit	(Ndoye *et al.*, 2007)
Acetobacter	*syzygii*	Fruit of Malay rose apple	(Lisdiyanti *et al.*, 2001)
Acetobacter	*sicerae*	Cider	(Carr, 1958) (Li *et al.*, 2014)
ACIDOMONAS			

Table 1.1. Currently established AAB genera and species and the isolation source of their type strain.

Genus	Species	Isolation source	Reference
Acidomonas	*methanolica*	Non-sterile yeast fermentation process	(Urakami *et al.*, 1989; Yamashita, 2004)
AMEYAMAEA			
Ameyamaea	*chiangmaiensis*	Flower of red ginger	(Yukphan *et al.*, 2009)
ASAIA			
Asaia	*bogorensis*	Orchid tree	(Yamada *et al.*, 2000)
Asaia	*astilbis*	*Astilbe*	(Suzuki *et al.*, 2010)
Asaia	*krungthepensis*	*Heliconia* flower	(Yukphan *et al.*, 2004a)
Asaia	*lannensis*	Flower of spider lily	(Malimas *et al.*, 2008b)
Asaia	*platycodi*	Balloon flower	(Suzuki *et al.*, 2010)
Asaia	*prunellae*	Flower of *Prunella vulgaris*	(Suzuki *et al.*, 2010)
Asaia	*siamensis*	Crown flower	(Katsura *et al.*, 2001)
Asaia	*spathodeae*	Flower of the African tulip	(Kommanee *et al.*, 2010)
BOMBELLA			
Bombella	*intestini*	Bomble bee crop	(Li *et al.*, 2015)
ENDOBACTER			
Endobacter	*medicaginis*	Surface-sterilized alfalfa	(Ramirez-Bahena *et al.*, 2013)
GLUCONACETOBACTER			
Gluconacetobacter	*aggeris*	Soil	(Nishijima *et al.*, 2013)
Gluconacetobacter	*liquefaciens*	Dried persimmon	(Yamada *et al.*, 1997)
Gluconacetobacter	*asukensis*	Ceiling of a historical site	(Tazato *et al.*, 2012)
Gluconacetobacter	*azotocaptans*	Rhizosphere of coffee plant	(Fuentes-Ramirez *et al.*, 2001)
Gluconacetobacter	*diazotrophicus*	Sugarcane	(Yukphan *et al.*, 2004a)
Gluconacetobacter	*entanii*	Submerged high-acid industrial vinegar fermentation	(Schuller *et al.*, 2000)
Gluconacetobacter	*takamatsuzukensis*	Soil	(Nishijima *et al.*, 2013)

Table 1.1. Currently established AAB genera and species and the isolation source of their type strain.

Genus	Species	Isolation source	Reference
Gluconacetobacter	*tumulisoli*	Soil	(Nishijima *et al.*, 2013)
Gluconacetobacter	*johannae*	Rhizosphere of coffee plant	(Fuentes-Ramirez *et al.*, 2001)
Gluconacetobacter	*sacchari*	Leaf sheath of sugarcane	(Franke *et al.*, 1999)
Gluconacetobacter	*tumulicola*	Stone chamber of a historical site	(Tazato *et al.*, 2012)
GLUCONOBACTER			
Gluconobacter	*albidus*	Flower	(Yukphan *et al.*, 2004b)
Gluconobacter	*cerevisiae*	Fermenting lambic beer	(Spitaels *et al.*, 2014a)
Gluconobacter	*cerinus*	Cherry	(Yamada and Akita, 1984)
Gluconobacter	*frateurii*	Strawberry	(Mason and Claus, 1989)
Gluconobacter	*japonicus*	Fruit of Hinese bayberry	(Malimas *et al.*, 2009b)
Gluconobacter	*kanchanaburiensis*	Spoiled jackfruit	(Malimas *et al.*, 2009a)
Gluconobacter	*kondonii*	Stawberry	(Malimas *et al.*, 2007)
Gluconobacter	*nephelii*	Rambutan	(Kommanee *et al.*, 2011)
Gluconobacter	*oxydans*	Beer	(De Ley, 1961; Gosselé *et al.*, 1983b; Henneberg, 1897; Mason and Claus, 1989)
Gluconobacter	*roseus*	Kaki fruit	(Malimas *et al.*, 2008c)
Gluconobacter	*sphaericus*	Fresh grapes	(Malimas *et al.*, 2008a)
Gluconobacter	*thailandicus*	Flower of Indian cork tree	(Tanasupawat *et al.*, 2004)
Gluconobacter	*uchimurae*	Rakam fruit	(Tanasupawat *et al.*, 2011a)
Gluconobacter	*wancherniae*	Unknown seed	(Yukphan *et al.*, 2010)
GRANULIBACTER			
Granulibacter	*bethesdensis*	Lymph node of a granulomatous disease patient	(Greenberg *et al.*, 2006)
KOMAGATAEIBACTER			(Yamada *et al.*, 2012)

Table 1.1. Currently established AAB genera and species and the isolation source of their type strain.

Genus	Species	Isolation source	Reference
Komagataeibacter	*europaeus*	High acid vinegar fermentation	(Sievers *et al.*, 1992; Yamada *et al.*, 1997; Yamada *et al.*, 2012)
Komagataeibacter	*hansenii*	Vinegar	(Gosselé *et al.*, 1983b; Lisdiyanti, 2006; Yamada *et al.*, 1997)
Komagataeibacter	*intermedius*	Kombucha	(Boesch *et al.*, 1997; Yamada, 2000)
Komagataeibacter	*kakiaceti*	Kaki vinegar	(Iino *et al.*, 2012b)
Komagataeibacter	*kombuchae*	Kombucha tea	(Dutta and Gachhui, 2007)
Komagataeibacter	*maltaceti*	Malt vinegar	(Slapšak *et al.*, 2013)
Komagataeibacter	*medellinensis*	Fruit vinegar	(Castro *et al.*, 2013)
Komagataeibacter	*nataicola*	Nata de coco	(Lisdiyanti, 2006)
Komagataeibacter	*oboediens*	Vinegar fermentation	(Sokollek *et al.*, 1998; Yamada, 2000)
Komagataeibacter	*rhaeticus*	Apple	(Dellaglio, 2005)
Komagataeibacter	*saccharivorans*	Beet juice	(Lisdiyanti, 2006)
Komagataeibacter	*sucrofermentans*	Cherry	(Cleenwerck *et al.*, 2010; Toyosaki *et al.*, 1995)
Komagataeibacter	*swingsii*	Apple	(Dellaglio, 2005)
Komagataeibacter	*xylinus*	Mountain-ash berries	(Komagata *et al.*, 2014; Yamada *et al.*, 1997)
KOZAKIA			
Kozakia	*baliensis*	Palm brown sugar	(Lisdiyanti, 2002)
NEOASAIA			
Neoasaia	*chiangmaiensis*	Flower of red ginger	(Yukphan *et al.*, 2005)
NEOKOMAGATAEA			
Neokomagataea	*tanensis*	Flower	(Yukphan *et al.*, 2011)
Neokomagataea	*thailandica*	Flower	(Yukphan *et al.*, 2011)
NGUYENIBACTER			
Nguyenibacter	*vanlangensis*	Rhizosphere of Asian rice	(Vu *et al.*, 2013)
SACCHARIBACTER			

Table 1.1. Currently established AAB genera and species and the isolation source of their type strain.

Genus	Species	Isolation source	Reference
Saccharibacter	*floricola*	Pollen	(Jojima *et al.*, 2004)
SWAMINATHANIA			
Swaminathania	*salitolerans*	Wild rice	(Loganathan and Nair, 2004)
SWINGSIA			
Swingsia	*samuiensis*	Flower	(Malimas *et al.*, 2013)
TANTICHAROENIA			
Tanticharoenia	*sakaeratensis*	Soil	(Yukphan *et al.*, 2008)

1.1.2. Metabolites of AAB

AAB possess unique biochemical features. One of the most profound metabolic characteristics common to most AAB is the oxidation of ethanol to acetic acid. This two-step reaction is mainly catalyzed by alcohol dehydrogenase and aldehyde dehydrogenase, which are located at the outer surface of the cytoplasmic membrane (Azuma *et al.*, 2009; Illeghems *et al.*, 2013a; Prust *et al.*, 2005; Raspor and Goranovič, 2008). The active sites of these dehydrogenases are oriented towards the periplasm, therefore, the substances can be oxidized in the periplasmic space without entering the cytoplasm (Deppenmeier *et al.*, 2002; Komagata *et al.*, 2014; Prust *et al.*, 2005). First, ethanol is oxidized to acetaldehyde by alcohol dehydrogenase, and then aldehyde is oxidized to acetic acid by aldehyde dehydrogenase.

Acetobacter species are capable of producing acetic acid from ethanol efficiently, therefore, many of them were isolated from vinegar and cocoa bean fermentation, where ethanol is first produced from carbohydrates by yeasts and then further oxidized by acetic acid bacteria. *Acetobacter* strains can further oxidize acetic acid to CO_2 and H_2O through the tricarboxylic acid cycle (TCA). However this only occurs at ethanol depletion and seems to be an irreversible change in their metabolism, after which they are not able to oxidize ethanol anymore. In the presence of ethanol, this metabolic pathway is repressed (Adams and Moss, 2000; Raspor and Goranovič, 2008). However, strains of *Gluconobacter* are unable to do this due to an incomplete TCA cycle (Prust *et al.*, 2005; Raspor and Goranovič, 2008).

The oxidative capacity towards sugar is more profound in *Gluconobacter* than in *Acetobacter* (Komagata *et al.*, 2014). In *Gluconobacter oxydans*, glucose is oxidized to gluconate via glucono-δ-lactone, mostly by membrane-bound NAD(P)+-independent glucose dehydrogenase, and further oxidized to 2-/5- ketogluconic acid and 2,5-diketogluconic acid. In general, *Gluconobacter* strains produce higher levels of gluconic acid than *Acetobacter* (Attwood *et al.*, 1991; Raspor and Goranovič, 2008).

Aside from glucose, most AAB can oxidize a wide variety of other

sugars and sugar-alcohols. For instance, *G. oxydans* is frequently used in industry for its strong ability to oxidize D-sorbitol to L-sorbose. These two sugars are involved in the production of L-ascorbic acid (vitamin C) via the Reichstein process (Deppenmeier *et al.*, 2002). *G. oxydans* possesses sorbitol dehydrogenase which completely oxidizes D-sorbitol to L-sorbose following the Bertrand-Hudson rule: "polyols with a cis-arrangement of two secondary hydroxyl groups in D-configurations to the adjacent primary alcohol group are oxidized to the corresponding ketoses". Following the same rule, 2,3-butanediol can be converted to acetylmethylcarbinol as well (Adachi *et al.*, 2003; Cheldelin *et al.*, 1957; De Muynck *et al.*, 2007; Joshi, 1974; Kersters *et al.*, 1965).

The membrane-bound dehydrogenases of AAB are linked to the respiratory chain since they transfer electrons to ubiquinone, which functions as an electron donor for ubiquinol oxidases (Matsushita *et al.*, 1994). The respiratory chain of AAB consists of quinoprotein or flavoprotein periplasmic dehydrogenases and terminal ubiquinol oxidase (UOX) (Matsushita *et al.*, 1994; Matsushita *et al.*, 2004). *Gluconobacter oxydans* possesses a simple system consisting of a non-proton-pumping NADH:ubiquinone oxidoreductase and two ubiquinol oxidases but lacks a proton-translocating NADH:ubiquinone oxidoreductase (complex I) and a cytochrome c oxidase (complex IV). Therefore, the ability to translocate protons in the course of redox reactions is rather limited. The electrochemical proton gradient is used to generate ATP via an F1 F0 -type ATP synthase (Prust *et al.*, 2005; Raspor and Goranovič, 2008). In contrast, sequence analysis of *Acetobacter pasteurianus* 386B revealed the presence of genes coding for a proton-translocating nicotinamide nucleotide transhydrogenase, which serves the purpose of translocating protons across the cytoplasmic membrane. The NADH+H^+ that derives from transhydrogenase activity can then be reoxidized by complex I, which is also present in this organism (Illeghems *et al.*, 2013a). Either of these two organisms possesses complex IV (Illeghems *et al.*, 2013a; Prust *et al.*, 2005). A recent study on the respiratory chain of AAB suggested that AAB have acquired UOX genes from β- and γ-*Proteobacteria* by horizontal gene transfer, while losing almost all the cytochrome oxidase (COX) genes (Matsutani *et al.*, 2014). This change in the respiratory

chain may enable AAB to perform incomplete oxidations, which contribute to the rapid oxidation of a variety of sugars or sugar alcohols present in fruit or flowers, and the accumulation of high concentrations of acid products in their environment.

1.1.3. Applications of AAB

AAB in fermented food and beverage products

Vinegar People have benefited from AAB long before they were recognized as one of the most important agents involved in vinegar fermentation. Vinegar is a transparent liquid, colorless or the color of the raw material, or colored by caramel, with a prescribed content of acetic acid between 40 and 150 g acetic acid L^{-1} (Ebner and Sellmer, 1995). Worldwide, vinegar is used as flavoring agent, as a preservative and, in some countries, also as a healthy drink. It is unclear when vinegar was first produced (Mazza and Murooka, 2009). In Latin, *acetum* means sour or sharp wine, hence, vinegar was probably a result of a spoiled alcoholic beverage. Its production can be as long as wine making and therefore may date back to at least 10,000 BC (Raspor and Goranovič, 2008). Not considering the diluted chemically produced acetic acid, vinegar can be made from almost any fermentable carbohydrate source by a two-step fermentation process involving yeasts as the first agent, followed by AAB. The raw materials for vinegar production are manifold and are mostly of plant origin, with two exceptions, those produced from whey or honey (Solieri and Giudici, 2009). In general, the vinegar fermentation process includes the raw material preparation, the alcoholic fermentation, the acetous fermentation and, finally, the maturation or ageing. In some specific vinegars, other microorganisms, such as moulds and lactic acid bacteria, are also involved in the fermentation process (Solieri and Giudici, 2009). The majority of AAB species involved in vinegar production belongs to the genera *Acetobacter* and *Gluconacetobacter* as species from these two genera possess strong alcohol oxidation ability.

Cocoa bean fermentation Fermented cocoa beans are the basic material in chocolate manufacture. The fermentation is a complex process that starts after the removal of the cocoa beans and the pulp from the cocoa pods. Yeasts, lactic acid bacteria and acetic acid bacteria contribute to the organoleptic properties of the end-products, both beneficially as well as detrimentally (De Vuyst *et al.*, 2010; Papalexandratou *et al.*, 2013; Schwan and Wheals, 2004). This biological process is spontaneous, and therefore the microbial species present differ between batches and different geographic locations. Occasionally, a more controlled situation is required, and starter cultures are used; however, when applied in large scale, these generally are more challenging compared with the spontaneous bioprocesses as it relates to starter culture production and maintenance, inoculation and fermentation method and extra costs (Mozzi *et al.*, 2010). In the cocoa bean fermentation process, three phases can be considered, reflecting the environmental factors (temperature, pH and oxygen tension) (De Vuyst *et al.*, 2010). In the initial phase, the high carbohydrate concentrations and the low pH of the fresh cocoa pulp-bean mass, together with the anaerobic conditions due to compact packing of the cocoa pulp-bean mass in heaps or boxes, favor the growth of yeasts, which produce ethanol and reduce the viscosity of the pulp causing it to drain away. As more pulp drains away and air penetrates the fermenting mass, lactic acid bacteria develop (De Vuyst *et al.*, 2010; Papalexandratou, 2011). Further on in the fermentation, more pulp drains away, the aeration increases, alcohol accumulates and the temperature rises above 37 ℃. The proportion of yeasts declines rapidly, and thermotolerant AAB predominate. The pulp is stirred and drained, which further increases the level of aeration. The presence of oxygen and the low pH favor the growth of AAB, mostly *Acetobacter* spp. (De Vuyst *et al.*, 2010; Papalexandratou, 2011). Subsequently, AAB oxidize ethanol, formed by yeasts, into acetic acid. This enzymatic activity causes a further rise in temperature up to 50 ℃. Together with the acid produced, it leads to the death of the seed embryo as well as the end of the fermentation (Cleenwerck *et al.*, 2007; Schwan, 1998). AAB species of the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* were all detected and isolated during cocoa bean fermentations.

Kombucha Kombucha is a traditional tea beverage obtained from black or green tea fermented by a symbiotic mixed culture of yeasts, lactic acid bacteria and AAB, which together form the so-called tea fungus (Dufresne and Farnworth, 2000; Greenwalt *et al.*, 2000; Marsh *et al.*, 2014). Kombucha has a history of several thousands of years in the East, and later has spread through Russia and Eastern-Europe by trade routes. It is considered to have positive health effects such as lowering blood pressure, enhancing immunity and curing cancer, yet, none of these effects have been scientifically proven (Dufresne and Farnworth, 2000; Greenwalt *et al.*, 2000). Analysis of kombucha has revealed the presence of acetic, lactic and gluconic acids as major chemical compounds and a diverse range of flavor compounds, such as alcohols, aldehydes, ketones and amino acids (Dufresne and Farnworth, 2000; Teoh *et al.*, 2004). To prepare kombucha, tea leaves are added to boiling water and allowed to infuse for about 10 min; afterwards, the leaves are removed. Sucrose [5 to 15 %, (w/v)] is dissolved in the hot tea. After cooling to room temperature, tea is poured into a wide-mouthed clean vessel and is acidified by adding already prepared kombucha. The tea fungus is laid on the tea surface, and the vessel is carefully covered with a clean cloth. The mixture is then left to incubate at room temperature for 1 to 8 weeks, depending on the preferred acidity of the beverage. When the bioprocess is completed, the beverage is filtered and stored in capped bottles at 4 °C. The taste of the kombucha changes during mixed fermentation from a pleasant fruity sour-like flavor after a few days, to a mild vinegar-like taste after prolonged incubation. During the fermentation process, the yeasts convert sucrose into fructose and glucose and produce ethanol. AAB convert glucose to gluconic acid and ethanol to acetic acid. The presence of acetic acid and antimicrobial metabolites effectively reduce the presence of other bacteria, yeasts and filamentous fungi (Sreeramulu *et al.*, 2000). *A. aceti*, *A. nitrogenifigens*, *A. pasteurianus*, *Kom. xylinus*, *Kom. intermedius*, *Ga. hansenii* and *G. oxydans* have all been isolated from kombucha (Komagata *et al.*, 2014; Raspor and Goranovič, 2008). Cellulose-producing AAB such as *Kom. xylinus* synthesize a floating cellulose network, which enhances the association formed between the bacteria and the yeasts. Caffeine and

related xanthines of the tea infusion stimulate the cellulose formation by *Kom. xylinus* (Dufresne and Farnworth, 2000; Raspor and Goranovič, 2008).

Water Kefir Water kefir is a water-sucrose beverage that is fermented at room temperature by kefir grains for about 24 to 48 h to produce carbon dioxide and low concentrations of ethanol. Traditionally, figs or other dried fruits and lemon are added to provide additional minerals and flavors. The beverage is fermented via a symbiosis of bacteria and yeasts that are present in the kefir grains (Neve and Heller, 2002). Water kefir grains are mostly translucent and crystal-like in appearance, composed of dextran, and commonly passed on from househould to househould (Marsh *et al.*, 2013). Compared to other fermented beverages such as kombucha, water kefir is less studied and claimed health benefits are yet to be confirmed (Marsh *et al.*, 2013). Culture-based and culture-independent methods have been used to assess the microbial populations of water kefir, revealing that the bacterial component is comprised of a varied mixture of *Lactobacillus*, *Lactococcus*, *Leuconostoc* and *Acetobacter*, while the yeast component is most commonly identified as *Saccharomyces*, *Hanseniaspora/Kloeckera*, *Zygotorulaspora* and *Candida* (Gulitz *et al.*, 2011; Laureys and De Vuyst, 2014; Marsh *et al.*, 2013; Neve and Heller, 2002).

Biotransformation and cellulose production

Chemical products Biotransformation, also called bioconversion, refers to the use of biological catalysts to convert a substrate into a product in a limited number of enzymatic steps (Raspor and Goranovič, 2008). Nowadays, many food, pharmaceutical and detergent industries take advantage of biocatalysts. The ability of AAB to oxidize various substrates has long been known and has been applied to many fields. The enzymatic oxidation of primary alcohols for the production of aldehydes is attractive because it can be carried out under mild conditions that are also suitable for labile products (Raspor and Goranovič, 2008). One of the earliest biotransformations using AAB is the production of vinegar

from ethanol. *Gluconobacter* are generally more ketogenic than *Acetobacter* strains and can oxidize a broad range of alcohols, sugars, sugar acids and sugar alcohols; therefore, they are widely used in industry for the production of L-sorbose from D-sorbitol, L-ribulose from ribitol, L-erythrulose from *meso*-erythrol, D-tagatose from D-galactitol, phenylacetaldehyde and phenylacetate from 2-phenylethanol, dihydroxyaceton from glycerol, (R)-3-hydroxy-2-methyl propionic acid from 2-methyl-1,3-ropandiol, 3-dehydroshikimate from quinate, and acetoin and diacetyl from 2,3-butanediol (Komagata *et al.*, 2014; Raspor and Goranovič, 2008).

Cellulose Cellulose is a polysaccharide consisting of a linear chain of several hundreds to many thousands of β-1,4-linked D-glucose units (Crawford, 1981; Updegraff, 1969). In nature, cellulose is an important structural component of the primary cell wall of green plants, many forms of algae and oomycetes. Some bacterial species secrete it to form a biofilm matrix. In its primary composition, bacterial cellulose and cellulose produced by other organisms (plants, algea) are the same, but the former has some unique physical characteristics. Strains of *Kom. xylinus* are considered the most efficient bacterial cellulose producers (Raspor and Goranovič, 2008; Ross *et al.*, 1991). Cellulose produced by *Kom. xylinus* presents excellent properties such as transparency, tensile strength, fiber-binding ability, adaptability to the living body and biodegradability (Takai *et al.*, 1975). During biosynthesis, carbon compounds in the nutrition medium are used by bacteria, polymerized into linear β-1,4-glucan chains and secreted outside the cells through pores on the outer membrane. Subsequently, the β-1,4-glucan chains are assembled outside the cell in a precise, hierarchical process, where they first form subfibrils and then microfibrils, and finally, bundles of microfibrils. In static cultures, *Kom. xylinus* forms a thick cellulosic surface mat (pellicle). The unique 3-D structure of this thick gelatinous membrane consists of an ultrafine network of highly uniaxially oriented cellulose nanofibers (3-8 nm), resulting in a high cellulose crystallinity (60 % - 80 %) and an enormous mechanical strength (Brown and Saxena,

2007). In addition, the size of the microbial cellulose fibrils is about 100 times smaller than that of plant cellulose, which results in a vast surface area, displaying great elasticity, high wet strength and conformability (Bielecki *et al.*, 2005; Raspor and Goranovič, 2008).

1.2. AAB as insect symbionts

Insect symbiosis has been a hot research topic in recent years, because symbiotic microbes can affect many aspects of the host, including its evolutionary history, lifestyle and physiology (Moran, 2006). Till now, the main insect sources for AAB are bees, mosquitoes, fruit flies and sugarcane mealybugs, with likely more to be discovered in the near future (Crotti *et al.*, 2010; Lambert *et al.*, 1981). As most AAB species favor sugary or alcoholic niches, many of their insect hosts feed on sugar rich flowers or fruits.

Among all the insects that are now known to have AAB endosymbionts, bees were the first in which AAB were detected (White, 1962). Table 1.2 provides an overview of AAB species and their insects host.

The major habitat of AAB in insects is the insects' digestive tract, although they were also detected on body surfaces, salivary glands and reproductive organs (Crotti *et al.*, 2010; Komagata *et al.*, 2014). For instance, *Acetobacter* spp. were detected on the body surface of *Drosophila*, with a smaller-than-normal size (Ren *et al.*, 2007). The ability of AAB to form a viable but not culturable (VBNC) state enables them to survive in harsh conditions, but also reduces the chance of being detected by culture-dependent approaches (Crotti *et al.*, 2010; Millet and Lonvaud-Funel, 2000; Trček and Barja, 2014). In the insect digestive tract, the anterior hindgut region is the most densely inhabited part, as the partly digested food as well as products secreted by the Malpighian tubules, provide the most nutritious conditions for bacteria (Crotti *et al.*, 2010). The (micro)aerobic environment, low pH and diet-derived sugars create an environment suitable for AAB to flourish and reproduce. Many AAB insect symbionts are also able to produce polysaccharides, which may assist them to attach to insect epithelial

cells (Chouaia *et al.*, 2014; Crotti *et al.*, 2009; Crotti *et al.*, 2010). *Asaia* strains are often found associated with larvae and adults of the mosquito *Anopheles stephensi* (Komagata *et al.*, 2014). *Asaia* strains tagged with fluorescent proteins also logged in both male and female reproductive organs and salivary glands; including the testicles of the leafhopper *Scaphoideus titanus* and the male genital duct in mosquitoes (Favia *et al.*, 2007). *A. tropicalis* was found in a brown gelatinous matrix within the peritrophic membrane of the olive fruit fly *B. oleae* (Crotti *et al.*, 2009; Favia *et al.*, 2007; Komagata *et al.*, 2014). It is clear from Table 1.2 that one AAB species can colonize different insects, vice versa, one insect can harbor multiple AAB species.

Table 1.2. AAB and related insects hosts.

AAB	Insect host(s)	Reference(s)
Acetobacter sp.		
	Drosophila melanogaster	(Corby-Harris *et al.*, 2007; Cox and Gilmore, 2007; Ren *et al.*, 2007; Ryu *et al.*, 2008)
	Apis mellifera	(Babendreier *et al.*, 2007; Mohr and Tebbe, 2007)
	Bactrocera oleae	(Kounatidis *et al.*, 2009)
	Amazonides tabida	(Zouache *et al.*, 2009)
	Saccharicoccus sacchari	(Ashbolt and Inkerman, 1990)
Asaia sp.		
	Anopheles sp.	(Crotti *et al.*, 2009; Damiani *et al.*, 2008; Favia *et al.*, 2008)
	Aedes aegypti	(Crotti *et al.*, 2009)
	Scaphoideus titanus	(Crotti *et al.*, 2009; Marzorati *et al.*, 2006)
	Marietta leopardiana	(Matalon *et al.*, 2007)
	Pieris rapae	(Robinson *et al.*, 2010)
	Bombus terrestris/lucorum	(Li *et al.*, 2015)
Bombella		
	Bombus lapidarus	(Li *et al.*, 2015)
	Bombus bimaculatus	(Cariveau *et al.*, 2014)
	Apis mellifera	(Corby-Harris *et al.*, 2014)
	Apis dorsata	(Cariveau *et al.*, 2014; Corby-Harris *et al.*, 2007)
	Xylocopa californica	(Martinson *et al.*, 2011)
"Commensalibacter" sp.		
	Drosophila melanogaster	(Roh *et al.*, 2008; Ryu *et al.*, 2008)
	Bombus pascuorum	(Li *et al.*, 2015)
	Danaus sp.	(Servin-Garciduenas *et al.*, 2014)

Table 1.2. AAB and related insects hosts.

AAB	Insect host(s)	Reference(s)
Gluconacetobacter sp.		
	Drosophilia melanogaster	(Corby-Harris *et al.*, 2007; Cox and Gilmore, 2007; Ryu *et al.*, 2008)
	Apis mellifera	(Babendreier *et al.*, 2007; Jojima *et al.*, 2004; Mohr and Tebbe, 2006)
	Saccharicoccus sacchari	(Ashbolt and Inkerman, 1990; Franke *et al.*, 1999)
Gluconobacter sp.		
	Apis mellifera	(Babendreier *et al.*, 2007; Mohr and Tebbe, 2007)
	Drosophila melanogaster	(Corby-Harris *et al.*, 2007; Cox and Gilmore, 2007; Ren *et al.*, 2007; Ryu *et al.*, 2008)
	Saccharicoccus sacchari	(Ashbolt and Inkerman, 1990)
Saccharibacter floricola		
	Apis mellifera	(Mohr and Tebbe, 2007)

Researchers have shown that *Asaia* strains can be transmitted from the mother to offspring, undergo paternal transmission to the progeny and cross-colonize phylogenetically distant insects (Crotti *et al.*, 2009; Crotti *et al.*, 2010; Damiani *et al.*, 2008). *Asaia* strains also showed a horizontal transmission route with rapid colonization of the gut, salivary glands and reproductive organs (Crotti *et al.*, 2009; Gonella *et al.*, 2012).

1.3. Genome studies of AAB

Currently, 20 complete genome and about 57 draft genome sequences of AAB are publicly available (Table 1.3). The former include genomes of *Acetobacter pasteurianus* (Azuma *et al.*, 2009; Illeghems *et al.*, 2013a), *Gluconacetobacter diazotrophicus* (Bertalan *et al.*, 2009), *Gluconobacter oxydans* (Ge *et al.*, 2013; Prust *et al.*, 2005), *Granulibacter bethesdensis* (Greenberg *et al.*, 2007), *Komagataeibacter medellinensis* (Ogino *et al.*, 2011) and *Komagataeibacter xylinus* (Kubiak *et al.*, 2014).

The genome of *A. pasteurianus* IFO 3283-01 is composed of 2,907,495 bp. It comprises more than 280 transposons and 5 genes with hypermutable tandem repeats as common features, which indicates genetic instability. Its chromosome contains 2,628 open reading frames (ORFs). Its metabolic capacities and mechanisms towards stress tolerance were investigated (Azuma *et al.*, 2009). Two pathways of production of dihydroxyacetone phosphate from glycerol were identified; one is via dihydroxyacetone and is catalyzed by glycerol dehydrogenase, the other is via glycerol 3-phosphate and is catalyzed by glycerol kinase. Genes encoding membrane-bound glycerol dehydrogenase were not found. Genome analysis revealed that a single nonsynonymous nucleotide mutation in the glycerol kinase gene seemed to relate to sugar production, as well as affect polysaccharide production and its structural modification. The analysis also revealed that a 92-kb deletion and 3 single nucleotide mutations occurred in the genome during high-temperature adaptation, resulting in a smaller genome and thus yielding a survival advantage under stressful conditions, possibly due to faster replication or lower heat generation based on less burden in DNA replication (Azuma *et al.*,

2009).

The genome of *A. pasteurianus* strain 386B was also fully sequenced. *A. pasteurianus* 386B was originally isolated from a spontaneous cocoa bean heap fermentation. Its genome consists of a 2,818,679 bp chromosome and 7 plasmids. The whole genome sequence and comparative genome analyses revealed metabolic pathways, several strain specific genes, niche adaptations traits and mechanisms involved in tolerance towards various stress conditions that enabled this strain to be an ideal candidate starter culture for controlled cocoa-bean fermentation processes. In addition, the low number of transposases in the genome and the absence of complete phage genomes indicated that the strain is genetically more stable than other *A. pasteurianus* strains (Illeghems *et al.*, 2013a).

The whole genome sequence of the industrially important strain *G. oxydans* 621H was thoroughly investigated and has been used to gain insight into its overall metabolism as well as into specific biotechnological features. The reconstructed metabolic pathways illustrated the mechanisms of industrially important products derived from sugars and alcohols, and also revealed that the membrane-bound dehydrogenases are critical for the incomplete oxidation of biotechnologically important substrates (Prust *et al.*, 2005). *G. oxydans* 621H contains many membrane-bound dehydrogenases, which enable this organism to take advantage of various sugar or alcohols from the environment. The oxidation of substrates takes place in the cytoplasmic membrane, being catalyzed by a set of oxidoreductases. The efficiency of these enzymatic reactions allows *G. oxydans* to survive and thrive in mixed microbial populations. In addition, the formation of sugar acids decreases the pH, and enables this strain to out-compete other microorganisms. Moreover, it has a rather simple respiratory chain that accelerates this process, although it has a low energy-transducing efficiency, which results in a very low growth yield (Prust *et al.*, 2005). In *G. oxydans* H24, a strain showing a high L-sorbose productivity, genes responsible for the production of L-sorbose were identified after whole-genome sequencing analysis (Ge *et al.*, 2013).

The endophytic bacterium *Ga. diazotrophicus* is associated with

sugarcane plants. The genome of the type strain of this species, Pal5^T, is composed of a 3,944,163 bp chromosome and 2 plasmids (Bertalan *et al.*, 2009). In contrast to other sequenced endophytic bacteria, the genome of this strain contains as many as 190 transposable elements, which seem to be associated with a high number of horizontal gene transfer events. The fact that most of these genes are similar to genes of bacteria from the order *Rhizobiales*, suggests that rhizosphere soil might be the previous niche of *Ga. diazotrophicus*. Furthermore, gene clusters encoding a *gum*-like polysaccharide, which could allow this organism to penetrate the plant in the process of niche changing, were identified. The genome also possesses many features that enhance plant fitness, such as genes encoding for biological nitrogen fixation, phytohormones and biocontrol. In addition, type IV secretion systems, flagella, pili, chemotaxis, biofilm, capsular polysaccharide and some transport proteins were found in genomic islands, all of which could be related to bacteria-plant interactions. The information provided by the genome sequence analysis may contribute to an improved sugarcane crop production by manipulation of the bacteria-plant interactions, and to other biotechnological applications (Bertalan *et al.*, 2009).

Granulibacter bethesdensis CGDNIH1^T was isolated from lymph nodes of a patient who suffered from chronic granulomatous disease (CGD). The chromosome of this strain contains 2,708,355 bp and 2,437 putative ORFs, of which 1,470 share sequence similarity with *G. oxydans*, while the 967 other ORFs are unique to *Gr. bethesdensis*. Several methanol dehydrogenase genes were identified and suspected to originate from *Methylobacterium extorquens* based on BLAST hits. The DNA uptake system in *Gr. bethesdensis* may have facilitated horizontal transfer of genes from *Methylobacterium* or others. Some virulence related ORFs may have been acquired the same way. Comparative analysis of the *Gr. bethesdensis* CGDNIH1^T genome to those of other known CGD pathogens demonstrated conservation of some putative virulence factors, indicating possible common mechanisms involved in pathogenesis in CGD.

The genome of the non-cellulose producing strain *Kom. medellinensis* NBRC 3288^T was sequenced in 2011 (Ogino *et al.*, 2011). This strain was

isolated from vinegar in Japan and was originally classified as *G. xylinus* (now *Kom. xylinus*). The complete genome consists of a single circular chromosome of 3,136,818 bp and 7 distinct plasmids. Genome annotation predicted 3,195 ORFs, of which putative functions were assigned to 2,358 genes and to 837 hypothetical genes. The genome analysis identified 11 genes related to cellulose synthesis within two operons. However, two genes encoding the cellulose synthase catalytic subunit were annotated as fragmented genes. A nonsense mutation that caused the split up of these two genes might affect the cellulose synthesis of this strain. Comparative analysis of the NBRC 3288^{T} genome sequence with those of cellulose-producing strains also identified genes that are important for cellulose production in the genus *Komagataeibacter*.

In addition to the genomes sequences discussed above, many other complete or draft genome sequences of AAB have been determined to expand the knowledge of various aspects of AAB biology, such as their phylogeny, physiology and ecology (Matsutani *et al.*, 2011; Matsutani *et al.*, 2013; Sakurai *et al.*, 2011; Sakurai *et al.*, 2013; Soemphol *et al.*, 2011). For instance, some of these sequences have been used to reconstruct a whole genome-based phylogeny of the genera *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (Matsutani *et al.*, 2010), while a study based on whole-genome sequence analysis of *A. pasteurianus* strains differing in thermotolerance revealed also phylogenetic differences among these strains (Matsutani *et al.*, 2011). Finally, Chouaia and co-workers compared genomes of *As. platycodi* and *Saccharibacter* sp. to reconstruct their phylogeny and to identify genes that enable AAB to establish symbiotic relationships with insects (Chouaia *et al.*, 2014).

Table 1.3. List of AAB strains with publicly available genome sequences at the time of writing [NCBI genome database (`http://www.ncbi.nlm.nih.gov/genome/`), June 2015].

Species	Strain	GenBank assembly accession	Assembly level
Acetobacter aceti	ATCC 23746	*GCA*_000379545.1	Scaffold
Acetobacter aceti	NBRC 14818^T	*GCA*_000193495.1	Contig
Acetobacter aceti	NBRC 14818^T	*GCA*_000963905.1	Contig
Acetobacter aceti	1023	*GCA*_000691125.1	Contig
Acetobacter cibinongensis	4H-1^T	*GCA*_000963925.1	Contig
Acetobacter indonesiensis	5H-1^T	*GCA*_000963945.1	Contig
Acetobacter malorum	DmCS_005	*GCA*_000743885.1	Contig
Acetobacter nitrogenifigens	DSM 23921	*GCA*_000429165.1	Scaffold
Acetobacter okinawensis	JCM 25146^T	*GCA*_000613865.1	Contig
Acetobacter orientalis	21F-2^T	*GCA*_000963965.1	Contig
Acetobacter orleanensis	JCM 7639^T	*GCA*_000964205.1	Contig
Acetobacter papayae	JCM 25143^T	*GCA*_000613285.1	Contig
Acetobacter pasteurianus	IFO 3283-01	*GCA*_000010825.1	Complete
Acetobacter pasteurianus	IFO 3283-03	*GCA*_000010845.1	Complete
Acetobacter pasteurianus	IFO 3283-07	*GCA*_000010865.1	Complete
Acetobacter pasteurianus	IFO 3283-22	*GCA*_000010885.1	Complete
Acetobacter pasteurianus	IFO 3283-26	*GCA*_000010905.1	Complete
Acetobacter pasteurianus	IFO 3283-32	*GCA*_000010925.1	Complete
Acetobacter pasteurianus	IFO 3283-01-42C	*GCA*_000010945.1	Complete
Acetobacter pasteurianus	IFO 3283-12	*GCA*_000010965.1	Complete
Acetobacter pasteurianus	386B	*GCA*_000723785.1	Complete
Acetobacter pasteurianus	NBRC 101655	*GCA*_000241585.2	Contig
Acetobacter pasteurianus	3P3	*GCA*_000285315.1	Contig
Acetobacter pasteurianus	LMG 1262^T	*GCA*_000241625.2	Contig
Acetobacter persici	JCM 25330^T	*GCA*_000613905.1	Contig
Acetobacter pomorum	DM001	*GCA*_000193245.2	Contig
Acetobacter pomorum	DmCS_004	*GCA*_000755675.1	Contig
Acetobacter syzygii	9H-2 ^{T}	*GCA*_000964225.1	Contig
Acetobacter tropicalis	DmCS_006	*GCA*_000755665.1	Contig
Acetobacter tropicalis	NBRC 16470^T	*GCA*_000787635.1	Contig
Acetobacter tropicalis	NBRC 101654	*GCA*_000225485.1	Contig
Acidomonas methanolica	NBRC 104435	*GCA*_000617865.1	Contig
Asaia astilbis	JCM 15831^T	*GCA*_000613845.1	Contig
Asaia platycodi	JCM 25414^T	*GCA*_000614545.1	Contig

Table 1.3. List of AAB strains with publicly available genome sequences at the time of writing [NCBI genome database (`http://www.ncbi.nlm.nih.gov/genome/`), June 2015].

Species	Strain	GenBank assembly accession	Assembly level
Asaia platycodi	SF2.1	*GCA*_000724025.1	Contig
Asaia prunellae	JCM 25354^{T}	*GCA*_000613885.1	Contig
Asaia sp.	SF2.1	*GCA*_000505765.1	Contig
"Commensalibacter intestini"	A911 ^{T}	*GCA*_000231445.2	Contig
"Commensalibacter" sp.	MX01	*GCA*_000527695.1	Contig
Gluconacetobacter diazotrophicus	PAL 5^{T}	*GCA*_000067045.1	Complete
Gluconacetobacter diazotrophicus	PAL 5^{T}	*GCA*_000021325.1	Complete
Gluconacetobacter sp.	SXCC-1	*GCA*_000208635.2	Contig
Gluconobacter frateurii	NBRC 101659	*GCA*_000284875.1	Contig
Gluconobacter frateurii	M-2	*GCA*_000964445.1	Contig
Gluconobacter frateurii	NBRC 103465	*GCA*_000509445.1	Scaffold
Gluconobacter morbifer	G707^{T}	*GCA*_000234355.2	Contig
Gluconobacter oxydans	621H	*GCA*_000011685.1	Complete
Gluconobacter oxydans	H24	*GCA*_000311765.1	Complete
Gluconobacter oxydans	DSM 3504	*GCA*_000583855.1	Complete
Gluconobacter oxydans	WSH-003	*GCA*_000263255.1	Scaffold
Gluconobacter oxydans	DSM 2003	*GCA*_000507285.1	Contig
Gluconobacter oxydans	NL71	*GCA*_001008185.1	Contig
Gluconobacter thailandicus	NBRC 3255	*GCA*_000344115.1	Contig
Gluconobacter thailandicus	NBRC 3257	*GCA*_000576285.1	Contig
Gluconobacter thailandicus	F149-1	*GCA*_000964465.1	Contig
Granulibacter bethesdensis	CGDNIH1^{T}	*GCA*_000014285.1	Complete
Granulibacter bethesdensis	CGDNIH3	*GCA*_000576085.1	Complete
Granulibacter bethesdensis	CGDNIH4	*GCA*_000576145.1	Complete
Granulibacter bethesdensis	CGDNIH2	*GCA*_000576185.1	Complete
Komagataeibacter europaeus	LMG 18494	*GCA*_000227545.1	Contig
Komagataeibacter europaeus	LMG 18890^{T}	*GCA*_000285295.1	Contig
Komagataeibacter europaeus	5P3	*GCA*_000285335.1	Contig

Table 1.3. List of AAB strains with publicly available genome sequences at the time of writing [NCBI genome database (`http://www.ncbi.nlm.nih.gov/genome/`), June 2015].

Species	Strain	GenBank assembly accession	Assembly level
Komagataeibacter europaeus	NBRC 3261	*GCA*_000964485.1	Contig
Komagataeibacter hansenii	ATCC 23769	*GCA*_000164395.1	Chromosome
Komagataeibacter hansenii	JCM 7643 ^{T}	*GCA*_000964405.1	Contig
Komagataeibacter intermedius	AF2^{T}	*GCA*_000817255.1	Scaffold
Komagataeibacter intermedius	TF2	*GCA*_000964425.1	Contig
Komagataeibacter kakiaceti	JCM 25156^{T}	*GCA*_000613305.1	Contig
Komagataeibacter medellinensis	NBRC 3288^{T}	*GCA*_000182745.1	Complete
Komagataeibacter oboediens	174Bp2	*GCA*_000227565.1	Contig
Komagataeibacter rhaeticus	AF1	*GCA*_000700985.1	Scaffold
Komagataeibacter xylinus	E25	*GCA*_000550765.1	Complete
Komagataeibacter xylinus	NBRC 13693	*GCA*_000964505.1	Contig
Kozakia baliensis	SR-745	*GCA*_000697575.1	Contig
Saccharibacter floricola	DSM 15669^{T}	*GCA*_000378165.1	Scaffold
Saccharibacter sp.	AM169	*GCA*_000723565.1	Contig
Tanticharoenia sakaeratensis	NBRC 103193	*GCA*_000963885.1	Contig

2
AAB taxonomy

Taxonomy is the science of defining groups of biological organisms on the basis of shared characteristics and giving names to those groups. Its main goal is to provide a reference system for biological knowledge. For bacteria, taxonomy is an important tool to give a clear phylogenetic picture of microorganisms to scientists, researchers and biotechnological industries.

2.1. History of AAB taxonomy

In the past one and half century, the taxonomy of AAB has been evolving along with the continuous development of bacterial systematics. The first acetic acid bacteria isolates date back to 1837, when F. T. Kutzing obtained the organisms from naturally fermented vinegar and named it *Ulvina aceti* (quoted in Asaia, 1968). The first description of AAB originates before 1900, when Louis Pasteur described the "mother of vinegar" in a study on vinegar (Gullo and Giudici, 2009). Later in 1898, Beijerinck introduced the name *Acetobacter*.

In the first half of the 20th century, the physiology and ecology of bacteria explored which started to impact on taxonomy. For a long time, the classification of bacteria was mainly based on their morphology, physiology and biochemical characteristics. In 1968, AAB were divided into two genera, *Acetobacter* and *Gluconobacter*, based on their capacity to oxidize ethanol and glucose, as well as on their flagellation pattern. The genus *Acetobacter* included microorganisms with peritrichous flagella (if motile) and capable to oxidize ethanol strongly, glucose weakly or not at all, and acetate and lactate completely to CO_2 and H_2O. Microorganisms with polar flagella (if motile), capable to oxidize glucose strongly, ethanol weakly or not at all, and unable to oxidize acetate and lactate to CO_2 and H_2O were classified into the genus *Gluconobacter*. Species and subspecies of each genus could be differentiated on the basis of phenotypic features (De Ley and Frateur, 1974a; De Ley and Frateur, 1974b).

In the years following the discovery of the double helical structure of the DNA molecule by James Watson and Francis Crick (Watson and Crick, 1953a; Watson and Crick, 1953b), molecular techniques based on DNA greatly facilitated the development of bacterial systematics. In 1961 DNA-DNA hybridization was proposed by McCarthy and Bolton as a powerful tool to distinguish closely related species. By the late 1970s, 16S rRNA gene sequence based phylogeny was proposed by Carl Woese and co-workers. In the mean time, numerical analysis of phenotypic features and protein based analyses were implemented into bacterial taxonomy and in the description of new species. The term "polyphasic" taxonomy was introduced to refer to the more rational way of species description, which considered both phenotypic and genetic characterizaton as well as chemotaxonomic characterization.

A great contribution to AAB taxonomy has been made by the research of De Ley and co-workers. In 1961, a comprehensive study of the oxidative behavior of AAB on several substrates was published by De Ley (De Ley, 1961). Two years later, De Ley and Schell (1963) studied the base composition of AAB DNA, which suggested a close relationship and a possible common phylogenetic origin of *Acetobacter* and *Gluconobacter*. In the beginning of the 1980s, an extensive reinves-

tigation of AAB taxa was carried out, and included the incorporation of numerical analysis of phenotypic features and/or DNA techniques into species descriptions. *Acetobacter* and *Gluconobacter* were united in the family *Acetobacteraceae* on the basis of rRNA-DNA hybridization data supported by phenotypic data (Gillis & De Ley, 1980). In the same year, strains that were previously identified as *Acetobacter aurantius* by Kondo and Ameyama (1958) were re-examined by Swings and co-workers. Based on the results of rRNA-DNA hybridization, these strains were removed from *Acetobacter* and *Gluconobacter*, and even from the rRNA "superfamily" to which these genera belonged. The latter rRNA superfamily was later shown to correspond with the *Alphaproteobacteria* (Stackebrandt *et al.*, 1988). A new genus *Frateuria* was proposed, with *Frateuria aurantia* as the type strain. In the same period more than 200 *Acetobacter* and *Gluconobacter* strains were investigated by numerical analysis of 177 phenotypic features and resulted in several taxonomic changes (Gosselé *et al.*, 1983a; Gosselé *et al.*, 1983b). In 1984, Yamada and Kondo divided the genus *Acetobacter* into two subgenera, *i.e.* the subgenus *Acetobacter* characterized by ubiquinone Q-9 and the subgenus *Gluconacetobacter* characterized by ubiquinone Q-10. The latter subgenus *Gluconacetobacter* was elevated to the genus level on the basis of partial 16S rRNA sequence analysis by Yamada and co-workers in 1997 (Yamada *et al.*, 1997).

From the mid-1980s onwards, DNA-DNA relatedness studies on AAB were conducted and several novel species were described on the basis of polyphasic taxonomic studies. In 1984 and 1985, several research groups re-investigated the taxonomy of the genus *Gluconobacter* by means of DNA-DNA hybridization experiments, which revealed that there were at least 3 genospecies within *Gluconobacter* (Micales *et al.*, 1985; Yamada *et al.*, 1984). Subsequently, these results led to the description of *Gluconobacter cerinus*, *Gluconobacter frateurii* and *Gluconobacter asaii*, and the emended the description of *Gluconobacter oxydans* (Mason and Claus, 1989; Yamada and Akita, 1984). At the end of the 1980s, a novel species *Acetobacter diazotrophicus* was described for a group of AAB isolated from roots and stems of sugarcane (Gillis *et al.*, 1989).

Since 2000, many polyphasic taxonomic studies of AAB have been

performed to classify AAB from novel niches, leading to the description of several novel genera, novel species and new combinations. In 2000 and 2002, the taxonomic position of several *Acetobacter* species was further examined (Cleenwerck *et al.*, 2002; Lisdiyanti *et al.*, 2000). These studies confirmed the need for DNA-DNA relatedness determination to obtain a stable AAB taxonomy and nomenclature, and reported several novel species and new combinations. At present, AAB are classified into 18 genera and 85 species (Bacterial Nomenclature Up-to-date, DSMZ, June 2015).

2.2. Polyphasic taxonomy

The current polyphasic approach used in taxonomic studies of bacteria contains phenotypic, genotypic and phylogenetic information. The combination of different techniques allows a more comprehensive species description but meanwhile also complicates the interpretation of results in comparison with a monophasic approach. Fortunately, nowadays, the methods applied are more or less standardized, making the interpretation more efficient. With the development of technology in different research fields, there is a growing number of techniques available: some of them are essential, some are interchangable with each other, and some are applicable but not necessary. Therefore it is important to choose the most appropriate strategy combining different techniques in practice.

2.2.1. Phenotypic analysis

Phenotypic analysis is the most classical identification approach for bacteria. Classical phenotypic analyses include investigation of the cell and colony morphology, and of physiological and biochemical features, such as information on growth temperature, pH range, and atmospheric conditions; growth in the presence of various substances and activity of a variety of enzymes. These characteristics often constitute the basis for the formal description of taxa from species to family level. For revealing genetic relatedness, many phenotypic features have been shown to be

irrelevant, but as a whole, they provide descriptive information enabling us to recognize taxa (Vandamme *et al.*, 1996).

Nevertheless, species level identification of AAB based on phenotypic information only is not recommended for several reasons. First of all, some AAB species such as *Acetobacter tropicalis*, *Acetobacter orleanensis* and *Acetobacter indonesiensis* can not be distinguished by phenotypic characteristics (Lisdiyanti *et al.*, 2000). Secondly, some strains are difficult to cultivate on artificial media and are therefore difficult to describe using phenotypic features (Lisdiyanti *et al.*, 2000; Yamada *et al.*, 1997). Thirdly, from a technical point of view, highly standardized procedures are required to obtain reproducible results within and between laboratories. Contradictory phenotypic data have been reported in different studies which may have been due to small differences in the composition of the growth media or the methods used (Spitaels *et al.*, 2014a). Finally, spontaneous mutations often occur in AAB. Strains can lose or gain phenotypic features such as acid resistance, ethanol oxidizing abilities or the ability to produce cellulose or pigments due to sequence insertions, deletions or horizontal gene transfer (Beppu, 1993; Leisinger *et al.*, 1967). For these reasons, it has been advised to use genotypic data as basis for species level identification of AAB (Cleenwerck and De Vos, 2008).

2.2.2. Chemotaxonomic analysis

Chemotaxonomy refers to methods that classify organisms based on differences and similarities in chemical markers such as lipids, proteins, or other cell constituents. The methods involved include determination of the isoprenoid quinone through high performance liquid chromatography, gas chromatographic separation of cellular fatty acid methyl esters (FAME) analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis of the whole cell proteins, Fourier transform infrared spectroscopy of bacterial infrared fingerprinting, Raman spectroscopy of bacterial cellular composition and Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of bacterial protein analysis. Among the above-mentioned meth-

ods, quinone and FAME analysis have been widely used to characterize AAB. However, the taxonomic value of these methods is limited. Most of the known AAB strains contain Q-10 as major ubiquinone, except for strains of *Acetobacter* that possess Q-9 as major ubiquinone. In addition, the cellular fatty acid composition is strongly influenced by the cultivation conditions used (*i.e.* medium, temperature, time), which implies that only profiles of strains cultivated in the same conditions can be compared. This makes this technique not so suitable for AAB which require different cultivation media for optimal growth. Moreover, the taxonomic resolution of cellular fatty acid analysis is rather limited (Cleenwerck and De Vos, 2008). More recently, MALDI-TOF MS has proven to be a rapid, accurate and cost-effective method for bacteria identification. The use of MALDI-TOF MS for microbial identification will be discussed later in 2.3.

2.2.3. Genotypic analysis

Generally speaking, genotypic methods include fingerprint-based and sequence-based techniques (Emerson *et al.*, 2008).

rRNA sequence analysis

16S rRNA gene based phylogeny is the backbone of prokaryotic systematics (Yarza *et al.*, 2010; Yarza *et al.*, 2008). The gene occurs in all bacterial genomes and can easily be amplified through the presence of highly conserved signature sequences. Consequently, 16S rRNA gene sequence based comparisons have been and are still commonly used to determine the phylogenetic position of a new bacterial isolate and allocate it to a taxon. The method is suitable from the level of domains (starting at about 55 % similarity) to genera and in some cases moderately related species, *i.e.* below 98.65 % rRNA sequence similarity. Organisms sharing more 16S rRNA gene sequence similarity may belong to the same species; however it was noticed that some strains that share very similar or even identical 16S rRNA gene sequences can be diverse at the whole genome level. Therefore, in case more than 98.65 % rRNA

sequence similarity is found, DNA-DNA hybridization or equivalent experiments need to be performed for identification at the species level (Kim *et al.*, 2014).

A phylogenetic tree based on nearly complete 16S rRNA gene sequences revealing the relationships of all established AAB species is shown in Figure 2.1. and reveals that several clusters of AAB species, such as *Gluconobacter albidus*, *Gluconobacter cerevisiae*, *Gluconobacter kondonii* and *Gluconobacter sphaericus*, are closely related. The overall pairwise similarity of 16S rRNA gene sequences of AAB species ranges from 92.1 to 99.0 %. An alternative to 16S rRNA gene sequences are the 16S-23S rRNA ITS gene sequences, which have a higher discriminatory power. Phylogenies based on such sequences show consistency with 16S rRNA gene based phylogenies, but closely related species can much more often be differentiated (Castro *et al.*, 2013; Kommanee *et al.*, 2011; Tanasupawat *et al.*, 2009; Trček and Teuber, 2002).

DNA-DNA hybridization

Since 1987, whole genome DNA-DNA hybridization (DDH) has been playing a key role in bacterial taxonomy. Wayne and colleagues then defined a bacterial species as an taxon that generally includes strains sharing approximately 70 % or more DNA-DNA relatedness and with 5 ℃ or less δTm, which is the difference in the DNA melting temperature between homologous and heterologous DNA hybrids (Wayne *et al.*, 1987). In addition, phenotypic and chemotaxonomic features should agree with this definition. These authors in fact proposed that taxonomy should be determined by phylogeny and the latter by complete genome sequences. However, since at that time sequencing of genomes at large scale was not possible, a pragmatic species definition based on DNA-DNA hybridization data was proposed.

DNA-DNA hybridization experiments have been criticized for being cumbersome, difficult to implement and having a high experimental error. Discrepancies of values obtained via different or even the same approaches have indeed been noticed (Cleenwerck and De Vos, 2008). Because of this, it has been proposed to replace DNA-DNA hybridiza-

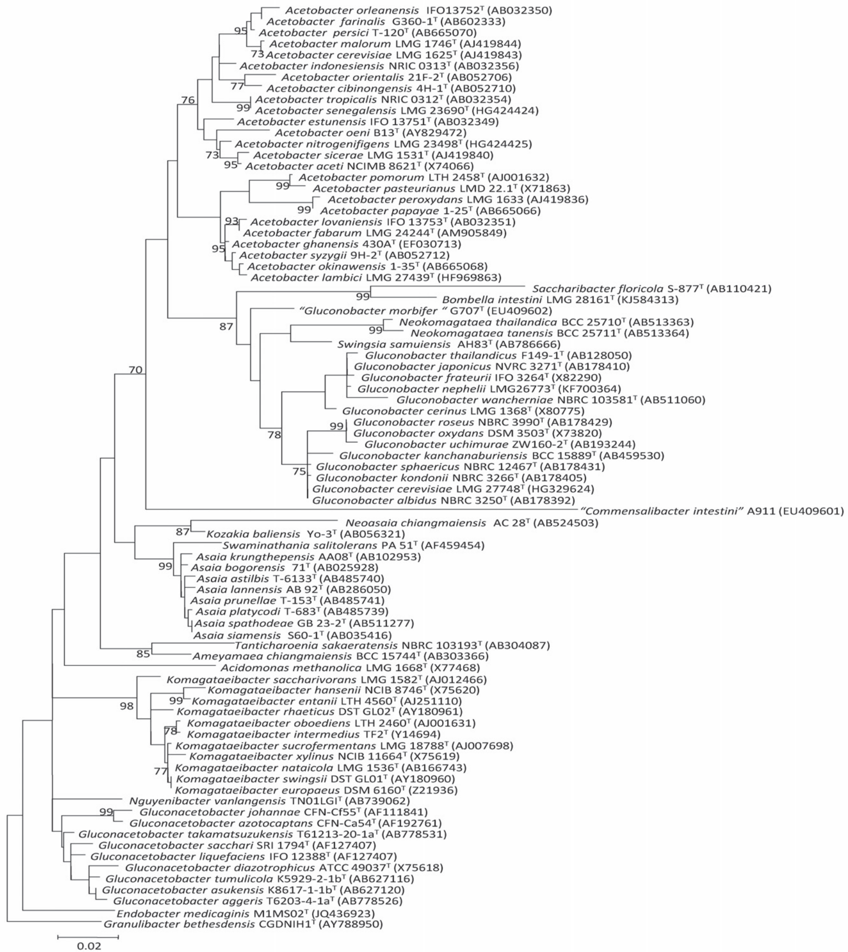


Figure 2.1. Maximum-likelihood tree based on nearly complete 16S rRNA gene sequences (1365 bp) of the type strains of all species of family *Acetobacteraceae* showing the phylogenetic relationships of this family. *Granulibacter bethesdensis* CGDNIH1^{T} was used as outgroup. The phylogenetic tree was constructed using MEGA 5. The DNA substitution K2+G+I was selected. The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets. Bar, 2 % sequence divergence.

tion experiments by equivalent alternative methods (Stackebrandt *et al.*, 2002). Multilocus-sequence analysis (MLSA) of concatenated housekeeping genes and calculation of average nucleotide identity (ANI) of shared genes between two genomes were reported promising techniques to overcome the problems associated with DNA-DNA hybridization experiments (Glaeser and Kämpfer, 2015; Konstantinidis and Tiedje, 2005; Stackebrandt *et al.*, 2002).

DNA-based fingerprinting methods

DNA based fingerprinting methods, such as restriction fragment length polymorphisms analysis (RFLP), amplified ribosomal DNA restriction analysis (ARDRA), random amplification of polymorphic DNA (RAPD) analysis, repetitive bacterial DNA (rep-PCR) fingerprinting, amplified fragment length polymorphism (AFLP) analysis and denaturing gradient gel electrophoresis (DGGE) of genomic macrorestriction fragments, have been widely used for the separation of species into a number of distinct types (Gullo and Giudici, 2009), while only a few of these techniques have been used for species level identification (Duim and Wagenaar, 2006; Yetiman and Kesmen, 2015). In taxonomic studies of AAB, AFLP and rep-PCR fingerprinting using the $(GTG)_5$-primer enabled species level identification when a robust reference database was available (Cleenwerck *et al.*, 2009; Papalexandratou *et al.*, 2009). Digestion of 16S-23S rRNA ITS spacer regions using restriction enzymes, such as *Hae*III, *Hpa*II, *Bsp*128I, *Mbo*II and *Ava*II, has also been used in several studies and revealed the existence of several new species, such as *Ga. saccharivorans*, *G. thailandicus*, *G. sphaericus*, *G. roseus* and *G. uchimurae*. Most of the other fingerprint techniques provided limited taxonomic information when applied to AAB (Cleenwerck and De Vos, 2008).

Nowadays, most of the DNA fingerprinting methods are being replaced by sequencing of housekeeping genes or by more rapid methods such as MALDI-TOF MS which reveals a protein fingerprint (see 2.3.2 and 2.3.3) (Huang *et al.*, 2014; Trček and Barja, 2014).

Multilocus sequence analysis (MLSA)

Multilocus sequence analysis (MLSA) uses the sequences of single-copy and widely occurring protein-coding genes and is currently a widely used method in bacterial taxonomy. Compared to 16S rRNA gene based phylogenies the phylogenetic relationships are depicted at a higher taxonomic resolution (Glaeser and Kämpfer, 2015). Protein-coding genes generally evolve faster than 16S rRNA genes and have because of this a better discriminatory power. Generally, MLSA based trees are similar but superior to 16S rRNA gene sequence based trees, as closely related species can often be differentiated. Analysis based on only one protein-coding gene is not recommended as it may not reflect the "true" phylogenetic relationships, because of the possibility of horizontal gene transfer or variable rates of mutations and recombinations (Gevers *et al.*, 2005). Analysis of (concatenated) sequences of at least three protein-coding genes has been advised for phylogenetic analyses (Konstantinidis *et al.*, 2006). For AAB taxonomy, the housekeeping genes *dnaK* (encoding a heat-shock protein), *groEL* (encoding a chaperonin protein) and *rpoB* (encoding the β-subunit of bacterial RNA polymerase) has widely been used (Cleenwerck *et al.*, 2010) (**Chapter 4**).

Determination of the DNA base composition (mol% G+C)

Determination of the DNA base composition (the mol % G+C content) is considered a part of the standard description of a bacterial taxon. The DNA G+C content does not provide phylogenetic information, but it has some discriminatory power. Within a well-defined species, the DNA G+C content is expected to differ with maximal 3 mol % (Vandamme *et al.*, 1996). Within the family *Acetobacteriaceae* the DNA G+C content ranges from 52 to 67 mol % (Cleenwerck and De Vos, 2008).

2.3. Identification of AAB using MALDI-TOF MS

2.3.1. Overview of MALDI-TOF MS

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique used in mass spectrometry for the analysis of biomolecules and large organic molecules (Hillenkamp and Karas, 2007; Liyanage and Lay, 2006). Although mass spectrometry has been used in chemistry for decades, its use in bacterial characterization was only proposed in 1975 (Anhalt and Fenselau, 1975). In this pioneer study, phospholipids and ubiquinones extracted from lyophilized bacteria were analysed, and genus specific or even species specific mass spectra were revealed. In the 1980s, other techniques such as plasma desorption, laser desorption and fast atom bombardment (FAB) were explored (Heller *et al.*, 1987; Ruelle *et al.*, 2004). The first proposal to use MALDI-TOF MS was in 1987 (Karas *et al.*, 1987), but only resulted in 1996 in spectra of protein biomarkers (Claydon *et al.*, 1996; Despeyroux *et al.*, 1996; Holland *et al.*, 1996; Krishnamurthy and Ross, 1996).

Generally, MALDI-TOF MS consists of 3 steps. First, a sample is mixed with a suitable matrix on a metal plate, allowing the crystallization of the sample within the matrix. Secondly, after loading the sample/matrix mixture into a mass spectrometer, a pulsed UV laser irradiates the sample, triggering the vaporization of the sample, which results in releasing ions of various sizes. Finally, the ions pass through accelerating grids and travel down a flight tube until they strike a detector at the end of the tube. The mass (m)/charge (z) ratios of ions reaching the detector is determined by precisely recording their time of flight (TOF) to reach it, with lighter ions reaching the detector faster than heavier ones. Besides, the number of desorbed ions of a particular size (intensity) is also measured. Based on all this info a mass spectrum can be generated, which typically reports m/z values on the x-axis and the intensity on the y-axis. (Liyanage and Lay, 2006).

2.3.2. MALDI-TOF MS in microbiology

MALDI-TOF MS has been proven useful for the characterization of a wide variety of microorganisms, including bacteria, fungi and viruses. Yet, most of the focus is on its applicability to bacteria. The drive to develop this technique was the need for a rapid, simple and cost-effective method for bacterial identification and source tracking, particularly for bacteria linked to public safety (Giebel *et al.*, 2010). During the past decade, MALDI-TOF MS has evolved quickly and led to a shift in the routine practice in clinical microbiology laboratories, where it is nowadays commonly used for identification of bacteria (Clark *et al.*, 2013; DeMarco and Ford, 2013; Nomura, 2015). Typically, novel isolates are identified by comparing their MALDI-TOF MS spectra to spectra of known bacteria contained in databases.

The most common MALDI-TOF MS approach in clinical microbiology laboratories is the identification of bacteria through the analysis of colonies picked up from solid media (Nomura, 2015). The usefulness of this approach for routine identification of clinical microorganisms has been shown in various studies. Of 1600 isolates analyzed by Seng and colleagues 84.1 % were correctly identified at the species level and 11.3 % only at the genus level (Seng *et al.*, 2009). In another study in which 980 clinical bacteria and yeasts were analyzed, the overall performance of MALDI-TOF MS was significantly better than that of conventional biochemical systems such as Vitek-II, API and biochemical tests (92.2 % and 83.1 %, respectively) (Veen *et al.*, 2010). Sogawa and collegues analysed 468 clinical bacterial strains belonging to 92 species through MALDI-TOF MS and evaluated the identifications obtained through comparison of the spectra against the libraries in the BioTyper 2.0 software. The identification success at the species and genus levels were 91.7 % and 97.0 %, respectively. In addition, MALDI-TOF MS identification was much faster than the conventional methods, as only 5 min was needed to obtain the result for a total of 468 strains of 92 species examined, whereas 5 to 48 hours would be needed to yield the same result through conventional methods (Sogawa *et al.*, 2011). Identification failures or misidentification have been reported and were

mainly associated with inappropriate sample preparations, an insufficient amount of spectra from suitable reference strains in the database or closely related species (Nomura, 2015; Seng *et al.*, 2009; Sogawa *et al.*, 2011; Veen *et al.*, 2010). Next to identification of cultured colonies, it has been reported that clinical specimens such as urine, cerebrospinal fluid and blood can be directly analyzed prior to cultivation (Bizzini and Greub, 2010; Ferreira *et al.*, 2010; La Scola and Raoult, 2009; Segawa *et al.*, 2014).

Besides the application of MALDI-TOF MS in clinical microbiology labs, the technique has been used to rapidly and accurately detect and identify food-borne pathogens, such as *Campylobacter* spp., *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Shigella flexneria*, *Staphylococcus aureus* and *Salmonella* spp. (Böhme *et al.*, 2010; Böhme *et al.*, 2012; Dieckmann *et al.*, 2008; Holland *et al.*, 2000). In addition, it was applied to distinguish between pathogenic and non-pathogenic contaminants in foods (Mazzeo *et al.*, 2006; Ochoa and Harrington, 2005) and has been shown useful in the detection and quantification of food or beverage spoilage bacteria (Kern *et al.*, 2013; Nicolaou *et al.*, 2012; Wieme *et al.*, 2014b), as well as beneficial bacteria involved in food fermentations, such as AAB and lactic acid bacteria (Andrés-Barrao *et al.*, 2013; De Bruyne *et al.*, 2011; Snauwaert *et al.*, 2013; Spitaels *et al.*, 2014a; Spitaels *et al.*, 2014b).

The application of MALDI-TOF MS to environmental samples has also been reported (Giebel *et al.*, 2008; Ruelle *et al.*, 2004; Siegrist *et al.*, 2007). With a standardized protocol for sample preparation, the identification of *E. coli*, *Salmonella* and *Acinetobacter* could be obtained by the observation of the respective genus-, species- and strain-specific biomarkers (Ruelle *et al.*, 2004). A study of bacterial source tracking compared the performance of MALDI-TOF MS and rep-PCR, and reported that MALDI-TOF MS offered an improved ability to correctly assign *E. coli* isolates to a specific source group, despite its lower repeatability. Therefore, it was suggested that it was a promising tool to address the problem of fecal contamination of surface waters (Siegrist *et al.*, 2007).

Reproducibility assessments are important, especially in the case

of library-based approaches. Various sample preparation methods, different organic solvent matrices and sample deposition methods have been investigated with the aim to obtain stable and reproducible mass spectra (Andrés-Barrao *et al.*, 2013; Böhme *et al.*, 2010; Dieckmann *et al.*, 2005; Kern *et al.*, 2013; Ruelle *et al.*, 2004; Williams *et al.*, 2003). Some experimental parameters were shown to have little to no effect on overall reproducibility, while others can have a significant impact. For example, cultivation conditions and sample preparation protocols can significantly affect spectrum quality and reproducibility (Sandrin and Demirev, 2014). The fact that cultivation media can have an effect is not surprising (Giebel *et al.*, 2010), as each type of medium provides bacteria with different nutrients, whereby bacteria in response to these nutrients may express different proteins (Ellwood and Tempest, 1972; Valentine *et al.*, 2005; Wunschel *et al.*, 2005). However, in some studies bacteria cultivated on different media yielded spectra showing only minor differences (Bernardo *et al.*, 2002; Conway *et al.*, 2001; Vargha *et al.*, 2006). As the conservative bacterial ribosomal protein dominant the MALDI-TOF MS spectra, it is expected that cultivation media would only have minor affect on the mass spectra profiles. Overviews of the current approaches, sample preparation methods, instruments and software tools were recently published (Clark *et al.*, 2013; Sandrin *et al.*, 2013).

2.3.3. Application of MALDI-TOF MS for AAB identification

The usefulness of MALDI-TOF MS for AAB identification has not yet been comprehensively studied. Andrés-Barrao *et al.* evaluated for the first time MALDI-TOF MS as method for AAB identification, using 64 *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (some were later transferred to *Komagataeibacter*) reference strains, and 48 isolates from superfacial and submerged semi-continuous acetification processes. The analysis using the SARAMIS™ software package resulted in a dendrogram that correlated well with phylogenies based on 16S rRNA gene sequences, with 4 species forming split clusters, namely, *A. peroxydans*,

Kom. xylinus (previously *Ga. xylinus*), *Kom. europaeus* (previously *Ga. europaeus*) and *G. cerinus*. All AAB isolates could be identified at the species level, and non-AAB isolates could be differentiated from AAB (Andrés-Barrao *et al.*, 2013).

Similarly, in a study on the applicability of MALDI-TOF MS for the identification of beer-spoiling AAB and LAB, 348 isolates retrieved from 14 spoiled beer and brewery samples were compared to databases with spectra of 273 AAB and LAB reference strains, representing potential beer spoilers belonging to 52 species. Peak-based numerical analysis enabled species identification of 327 (94 %) isolates. The remaining isolates were assigned through sequence analysis of protein coding genes to species not known as beer spoilers, and thus not present in the database, or to novel AAB species (Wieme *et al.*, 2014b). In addition, during the construction of the AAB reference database several AAB strains were revealed to be misclassified (Wieme *et al.*, 2014b). Wieme *et al.* also investigated the effects of growth media on the MALDI-TOF MS spectra generated (Wieme *et al.*, 2014a) and concluded that the growth medium effects do not disturb species level differentiation of AAB; yet they strongly affected the potential for strain level differentiation. In addition, they reported that identification databases comprising spectra of multiple reference strains per species grown on different culture media are expected to facilitate species and strain level differentiation. Finally, MALDI-TOF MS was shown capable to differentiate several recently described AAB species from their phylogenetic closest neighbours (Li *et al.*, 2015; Li *et al.*, 2014; Spitaels *et al.*, 2014a; Spitaels *et al.*, 2014b).

2.4. Genomic taxonomy

As reported above, Wayne and collegues proposed in 1987 that taxonomy should be determined by phylogeny and the latter by complete genome sequences. The rapid development of next generation sequencing methods rendered whole-genome sequencing (WGS) affordable and feasible in a relatively short period of time (Nakamura *et al.*, 2011). Therefore, it can be expected that WGS data will affect the bacterial

species definition in the near future, as it has already been reported that the species definition can be defined on the basis of genomic signatures (Thompson *et al.*, 2013a). Also, it can be foreseen that the wealth of new data will be used for a critical evaluation of the current taxonomic system (Kämpfer and Glaeser, 2012).

Whole genome sequences contain taxonomic information that can be extracted and used to establish a solid framework for the bacterial species identification and classification (Konstantinidis and Tiedje, 2005; Richter and Rossello-Mora, 2009; Thompson *et al.*, 2013a). Genomic taxonomy is based on an integrated comparative genomics approach, which can include supertree analysis, core and pan genome analysis, multilocus sequence analysis of core genes, calculation of average nucleotide identity (ANI) and average amino acid identity (AAI), *in silico* genome-genome distance hybridization (GGDH) and investigation of species-specific signatures, codon usage bias and metabolic pathways.

Phylogenetic trees can be based on total or partial genome comparisons, including concatenated sequences of all conserved genes of the core genome (*i.e.* supertrees) (Daubin *et al.*, 2001) or of selected genes. Inferring reasonable phylogenies from genome sequences was long expected to be difficult, particularly because of the role of lateral gene transfer (LGT) in microbial evolution. Yet, it became clear that trees based on WGS data confirmed the 16S rRNA gene based hierarchical structure at least at the genus level and above, and therefore it was concluded that the 16S rRNA gene based hierarchical structure could at least for the moment remain the backbone of prokaryotic systematics (Kämpfer and Glaeser, 2012). Nevertheless, Klenk and Göker (2010) reported that genome-scale data will improve microbial taxonomy considerably, once there is sufficient coverage of major lineages based on type strains and a more detailed insight into some processes such as LGT (Kämpfer and Glaeser, 2012; Klenk and Göker, 2010). ANI analysis of conserved and shared genes between two bacterial strains based on pair-wise genome comparisons has been shown useful to distinguish prokaryotic species and was reported a promising technique to replace DNA-DNA hybridization experiments (Konstantinidis and Tiedje, 2005). A similar conclusion was made using ANI values based on comparison of

genomes that were artificially cut into 1020 nucleotides fragments (Goris *et al.*, 2007). Later, it became clear that comparison of at least 20 % of the genomes sequence suffices to determine reliable ANI values (Richter and Rossello-Mora, 2009). In that study, a threshold of 95-96 % ANI was recommended to delineate bacterial species (Richter and Rossello-Mora, 2009). Recently, genome distances based on genome-to-genome sequence comparisons and calculated using an online tool named the Genome-To-Genome Distance Calculator (GGDC) were reported useful for species delimitation as well (Meier-Kolthoff *et al.*, 2013; Thompson *et al.*, 2013a).

Moreover, genomes have species-specific signatures, which reflect the di-, tri-, or tetranucleotide relative abundance and are more similar between closely related than between distantly related species (Bohlin and Skjerve, 2009; Karlin and Burge, 1995; Karlin *et al.*, 1997). Genome sequences can also contribute to prokaryotic genus delimitation. A study using the percentage of conserved proteins between two strains to estimate their evolutionary and phenotypic distance was carried out very recently and demonstrated that the percentage of conserved proteins can serve as a robust genomic index for establishing the genus boundary for prokaryotic groups, where two species belonging to the same genus would share at least half of their proteins (Qin *et al.*, 2014).

Finally, some phenotypic features can also be deduced from genome sequences by means of the analyses of metabolic pathways or the presence/absence of diagnostic genes (Thompson *et al.*, 2013b; Wang *et al.*, 2011).

The number of publicly available AAB genome sequences has increased in the past 10 years and several whole genome sequence based phylogenetic studies have been carried out. The phylogenetic relationships among *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* (now split into *Gluconacetobacter* and *Komagataeibacter*) was investigated by comparison of five complete genome sequences, *i.e. A. pasteurianus* IFO 3283-01, *Ga. diazotrophicus* PAl 5^T, *G. oxydans* 621H, *Gr. bethesdensis* CGDNIH1^T, and *Acidiphilium cryptum* JF-5 (Matsutani *et al.*, 2010). While phylogenetic analysis of *Acetobacteraceae* species based on 16S rRNA gene sequences suggested that *Gluconacetobacter* was the first

to diverge from the common ancestor of *Acetobacter*, *Gluconobacter* and *Gluconacetobacter*, phylogenetic analysis of metabolic proteins and orthologous genes suggested that *Gluconobacter* was the first to diverge from the common ancestor. The latter result seems more logical as *Acetobacter* and *Gluconacetobacter* share more similar habitats and more metabolic patterns (Matsutani *et al.*, 2010). In addition, later studies supported these findings. Illeghems *et al.* (2013) found that *Acetobacter* genomes had more genes in common with *Gluconacetobacter* genomes compared to *Gluconobacter* genomes, and more recently, a study with 14 AAB genomes revealed that a phylogenomic tree based on 70 CDSs supported the findings of Matsutani *et al.* (2011) (Chouaia *et al.*, 2014).

Part III.

Experimental Work

Preamble

In this part of the thesis, **experimental work** performed in the frame of this Ph.D thesis is presented. In **Chapter 3**, MALDI-TOF MS was applied to well-characterized AAB strains representing the family *Acetobacteraceae* to construct a database for quick identification of AAB and detection of novel AAB species. The database was evaluated using AAB isolates obtained by Papalexandratou and coworkers from spontaneous cocoa bean fermentations carried out in Ecuador, Brazil and Malaysia in the period 2007-2010 (Papalexandratou, 2011; Papalexandratou and De Vuyst, 2011; Papalexandratou *et al.*, 2013). $(GTG)_5$-PCR fingerprints had been generated for these isolates, but had not resulted in an identification at the species level after comparison of these fingerprints against a reference database. Using the MALDI-TOF MS database, all these isolates could be identified, and it was revealed that about half of them were assigned to recently described species. Further evaluation of the MALDI-TOF MS database was done using presumed or poorly characterized AAB strains from the BCCM/LMG Bacteria Collection. More than 90 % of these strains could be identified. The remaining strains were either representing novel species of the family or not AAB. Furthermore, a few taxonomic problems existing within the family *Acetobacteraceae* were confirmed through MALDI-TOF MS data and further investigated through modern whole-genome sequencing (WGS) analysis. This revealed the synonymy of *Gluconobacter oxydans* and *Gluconobacter uchimurae*, and of *Gluconobacter japonicus* and *Gluconobacter nephelii*. In **Chapter 4**, AAB obtained by Wieme and coworkers during a study of microorganisms present in traditionally produced kefir, were grouped on the basis of their MALDI-TOF MS profile with LMG 1530 and LMG 1531^{T}, and were classified as *Acetobacter* sp.

The MLSA scheme for the genus *Acetobacter* was completed, along with the description of *Acetobacter sicerae* sp. nov. through a polyphasic taxonomy approach. Using different isolation strategies and media, AAB were obtained from gut samples of bumble bees caught in Belgium by Praet and coworkers in the frame of a bumble bee gut microbiota study (Praet *et al.*, 2015). These AAB were analysed by MALDI-TOF MS, which indicated that they constituted a novel taxon. This led to the description of *Bombella intestini* gen. nov., sp. nov. by means of a polyphasic taxonomy approach (**Chapter 5**). One strain of this newly described genus, LMG 28161^{T}, was subjected to whole-genome sequence analysis and its metabolism investigated more in depth (**Chapter 6**).

3

Improved classification of acetic acid bacteria through MALDI-TOF MS: *Gluconobacter nephelii* and *Gluconobacter uchimurae* are the later heterotypic synonyms of *Gluconobacter japonicus* and *Gluconobacter oxydans*, respectively

Leilei Li, Luc De Vuyst, Ilse Cleenwerck, and Peter Vandamme

Submitted to Systematic and Applied Microbiology, 2015

Summary

Identification of acetic acid bacteria (AAB) based on phenotypic approaches is rather difficult and often not accurate. Molecular based methods, in contrast, are more straightforward and often allow species level differentiation. Using the latter methods, several species have been reclassified, emended or described in the last two decades. However, several closely related AAB species remain difficult to differentiate. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has previously been proven useful for the identification of a limited number of AAB species. In the present study, a MALDI-TOF MS database containing profiles of approximately 280 AAB reference strains, covering 17 genera and more than 80 species representing the family *Acetobacteraceae*, was constructed. The database was evaluated as tool for fast identification of AAB using 33 isolates from cocoa bean fermentations and a large set of poorly characterized AAB strains from the BCCM/LMG Bacteria Collection. The MALDI-TOF MS reference database clustered most of the reference strains according to their species designation. Only a few species were split into two clusters, while some closely related species formed intermixed clusters. Using this database, the majority of AAB isolates and poorly characterized BCCM/LMG AAB strains could be accurately identified, which was confirmed through AFLP DNA fingerprinting, MLSA or DNA-DNA hybridizations. A few *Gluconobacter* strains that could not be clearly identified were subjected to whole-genome sequencing together with the type strains of the phylogenetic nearest neighbor species. In addition, representatives of two intermixed clusters, namely *Acetobacter cerevisiae*/*Acetobacter malorum* and *Gluconobacter japonicus*/*Gluconobacter nephelii*, as well as strains of the closely related species *Acetobacter tropicalis* and *Acetobacter senegalensis*, were investigated. Average nucleotide identity (ANI) values were calculated and enabled to identify the *Gluconobacter* strains. The data also revealed that *A. cerevisiae* and *A. malorum* are distinct species, while *G. nephelii* and *G. uchimurae* were revealed as later heterotypic synonyms of *G. japonicus* and *G. oxydans*, respectively.

3.1. Introduction

The present classification of acetic acid bacteria (AAB) is the result of a scientific process during which new and improved identification technologies have continuously been introduced (Cleenwerck and De Vos, 2008; Komagata *et al.*, 2014). Traditionally, AAB identifications have been performed by studying physiological and chemotaxonomic properties, which are time-consuming and often unreliable. These methods have been complemented gradually or replaced by molecular identification methods such as rep-PCR, amplified fragment length polymorphism (AFLP) DNA fingerprinting and sequencing of 16S-23S ITS (internal transcribed spacer) regions or housekeeping gene (Cleenwerck *et al.*, 2009; De Vuyst *et al.*, 2008; Trček and Teuber, 2002). The use of these modern molecular methods resulted in great improvement of AAB classification and a relatively high rate of correct identifications (Cleenwerck *et al.*, 2010; Mason and Claus, 1989; Micales *et al.*, 1985). Still, these methods have drawbacks too, especially concerning the speed and cost to construct and keep up-to-date identification databases (Giebel *et al.*, 2008; Olive and Bean, 1999; Papaexandratou *et al.*, 2011), as well as to identify new isolates. Therefore, a more rapid, accurate and cost-effective method for AAB identification remains appealing (Giebel *et al.*, 2008; Seng *et al.*, 2009). Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) was introduced for bacterial identification more than 30 years ago (Anhalt and Fenselau, 1975; Claydon *et al.*, 1996; Krishnamurthy and Ross, 1996). Meanwhile, various studies have shown the usefulness of MALDI-TOF MS for species level identification of food-related bacteria (De Bruyne *et al.*, 2010; Dieckmann *et al.*, 2008; Holland *et al.*, 2000). Recently, MALDI-TOF MS fingerprinting has been used for the rapid identification of vinegar fermentation and beer spoilage acetic acid bacteria of the genera *Acetobacter*, *Gluconacetobacter*, *Komagataeibacter* and *Gluconobacter* (Andrés-Barrao *et al.*, 2013; Wieme *et al.*, 2014b).

In the present study, 279 well-characterized AAB strains representing the family *Acetobacteraceae* were used to construct a MALDI-TOF MS identification database. The database was validated using 33 AAB

isolates obtained in previous studies from spontaneous cocoa bean fermentations carried out worldwide, along with approximately 260 poorly characterized AAB strains from the BCCM/LMG Bacteria Collection. AFLP, multi-locus sequence analysis (MLSA) based on concatenated partial *dnaK*, *groEL* and *rpoB* gene sequences and DNA-DNA hybridizations were performed on a selection of strains to verify the accuracy of the results obtained. A few strains that could not be identified or with a doubtful classification were subjected to whole-genome sequencing for calculation of average nucleotide identity (ANI) values with the aim to classify them properly.

3.2. Material and methods

3.2.1. Strains

Two hundred and seventy-nine AAB strains used for the construction of the MALDI-TOF MS reference database were obtained from the following culture collections: BCCM/LMG, BCC, NBRC, DSMZ and Riken BRC, and the research collection of LM-UGent (Table S3.1). Most of these strains were identified in previous studies using AFLP or 16S-23S ITS analysis (Cleenwerck *et al.*, 2009; Tanasupawat *et al.*, 2009) and were obtained from various sources, geographic regions or times.

Eighty-one AAB isolates were obtained in previous studies from cocoa bean fermentations carried out in Ecuador, Brazil and Malaysia in the period 2007-2010 that could not be identified through comparison of their $(GTG)_5$-PCR DNA fingerprints against a reference database (De Vuyst *et al.*, 2008; Papalexandratou *et al.*, 2009). This method grouped the 81 isolates into 17 clusters (data not shown). Thirty-three representatives were selected to test the usefulness of the MALDI-TOF MS reference database for quick and accurate identification of AAB (Table 3.1).

In addition, 264 AAB strains from the BCCM/LMG Bacteria Collection were subjected to MALDI-TOF MS analysis (Table S3.2). These isolates originated from various sources and countries and were mainly

classified on the basis of phenotypic features several decades ago.

3.2.2. MALDI-TOF MS analysis

To choose a suitable medium for the construction of the MALDI-TOF MS database, 20 AAB strains representing the phylogenetic diversity within the family *Acetobacteraceae* were cultivated onto four different agar media. The selected strains were revived on LMG medium 13 [2.5 % (w/v) D-mannitol; 0.5 % (w/v) yeast extract (Oxoid, Basingstoke, UK); 0.3 % (w/v) bacteriological peptone (Oxoid, Basingstoke, UK) and 1.5 % (w/v) agar] and subsequently transferred to LMG medium 13, LMG medium 404 [5 % (w/v) D-glucose; 1 % (w/v) yeast extract (Oxoid) and 1.5 % (w/v) agar], acetic acid medium (AAM) agar [1 % (w/v) D-glucose; 1.5 % (w/v) bacteriological peptone (Oxoid); 0.8 % (w/v) yeast extract (Oxoid); 0.3 % (v/v) acetic acid; 0.5 % (v/v) ethanol; 0.32 % (v/v) hydrochloric acid and 1.5 % (w/v) agar (Lisdiyanti *et al.*, 2001)] and LMG medium 405 [5 % (w/v) D-glucose; 1.0 % (w/v) yeast extract (Oxoid); 1.0 % (v/v) acetic acid; 2.0 % (v/v) ethanol and 1.5 % (w/v) agar]. All strains were subcultivated for 5 generations at 28 °C and each generation was grown for 48-72 h. Based on the initial results, LMG medium 13 and LMG medium 404 were chosen as agar media for the cultivation of all strains for MALDI-TOF MS analysis. AAM agar and LMG medium 405 were used occasionally when strains showed no growth on either of the two former agar media.

From each strain, about 1 μg of cells was taken and suspended into a tube with 300 μL Milli-Q water and 900 μL ethanol. After centrifugation, the cell pellets were stored at -20 °C or directly used for extraction. Cell extracts were prepared as described previously (De Bruyne *et al.*, 2011).

For each sample, 1 μL of cell extract was spotted onto a 384-well stainless steel target plate in duplicate and air-dried. Subsequently, an equal volume of a matrix-organic solvent mixture [α-cyano-4-hydroxycinnamic acid (CHCA) (5mg/mL) in a 50:48:2 acetonitrile (ACN): water: trifluoroacetic acid (TFA) matrix solution] was added to the spots and allowed to dry in air. Afterwards, the sample plate was subjected to MALDI-TOF MS analysis (4800 Plus MALDI-TOF/TOF™ Analyzer,

AB Sciex, MA, USA). Measurements were performed in a linear mode at an acceleration voltage of 20 kV (De Bruyne *et al.*, 2011). The mass range was set from 2 to 20 kDa. For each spot, 2000 spectra were generated, collected and presented as one main spectrum. All profiles were evaluated by their number of peaks, their signal intensity and slope. Profiles with a minimum quality (signal intensity $>$ 500, slope $<$ 30 %, number of peaks $>$ 5) were imported into a BioNumerics v7.1 database (Applied Maths, Sint-Martens-Latem, Belgium) for further analysis. Preprocessing of the raw data involved trimming, baseline subtraction, smoothing, noise computing, and peak picking (Wieme *et al.*, 2012). The peak-based Dice coefficient and curve based Pearson product moment correlation coefficient were used to calculate profile similarities. Dendrograms were constructed using the un-weighted paired-group method with arithmetic means (UPGMA) (Wieme *et al.*, 2014a).

3.2.3. 16S rRNA gene sequence analysis, AFLP, MLSA and DNA-DNA hybridizations

16S rRNA gene sequence analysis, AFLP, MLSA based on concatenated partial *dnaK*, *groEL* and *rpoB* gene sequences or DNA-DNA hybridizations using a modification of the method described by Ezaki (Ezaki *et al.*, 1989) were performed on selected strains, as described previously (Castro *et al.*, 2013; Cleenwerck *et al.*, 2002; Snauwaert *et al.*, 2013). DNA-DNA hybridizations were performed under stringent conditions at 46 ℃ in a solution containing 50 % (v/v) formamide. For every DNA pair, quadruplicate reciprocal reactions were carried out. Sequences were compared with sequences taken from the NCBI database (http://www.ncbi.nlm.nih.gov). Phylogenetic trees were constructed with the MEGA 6 software package (Tamura *et al.*, 2013) using the maximum-likelihood and neighbor-joining methods (Felsenstein, 1981; Saitou and Nei, 1987). Bootstrap values based on 1000 replicates were calculated.

3.2.4. Whole-genome sequencing (WGS), assembly and calculation of ANI values

Genomic DNA for WGS was extracted using the method of Wilson as modified previously (Cleenwerck *et al.*, 2002). The integrity and purity of the DNA were evaluated on 1.0 % (w/v) agarose gels and by spectrophotometric measurements at 234, 260 and 280 nm, respectively. A Quantus™ fluorometer and a QuantiFluor®ONE ds DNA system kit (Promega Corporation, Madison, WI, USA) were used to estimate the DNA concentration. Library preparation and genome sequencing were performed by BaseClear BV (Leiden, The Netherlands). Paired-end sequence reads were generated using the Illumina HiSeq2500 system (Illumina Inc., San Diego, CA, USA).

The initial *de novo* genome assembly of the raw reads was performed using the CLC Genomics Workbench v7.5 (CLC Inc., Aarhus, Denmark). For each genome, a *.fasta* file containing contigs longer than 1000 bp were extracted and used for ANI calculation. The ANIs between sequenced genomes were calculated using an in-house developed python pipeline based on JSpecies (Richter and Rossello-Mora, 2009). The MUMmer (NUCmer) algorithm was used to align the input sequences (Kurtz *et al.*, 2004; Richter and Rossello-Mora, 2009).

3.2.5. Phenotypic tests

Acid production from melibiose, L-sorbose and maltose, and growth on maltose as the sole carbon source were verified as reported previously (Gosselé *et al.*, 1983b).

3.3. Results

3.3.1. Medium selection

Most of the 20 strains cultivated on the four selected agar media grew well on LMG medium 13 and LMG medium 404, while acetified agar media with ethanol such as AAM agar and LMG medium 405 were

suitable for cultivating strains of *Acetobacter*, but not for strains of *Asaia* or *Gluconobacter*. Comparisons of the MALDI-TOF MS profiles from these strains revealed overall more homogeneous clusters if the profiles originated from cells cultivated on the same agar medium. To construct a robust identification database that would cover variations caused by the growth medium, all 279 strains were subsequently cultivated on LMG medium 13 and LMG medium 404. Only when no or very weak growth was found on one of these media, AAM agar or LMG medium 405 were used.

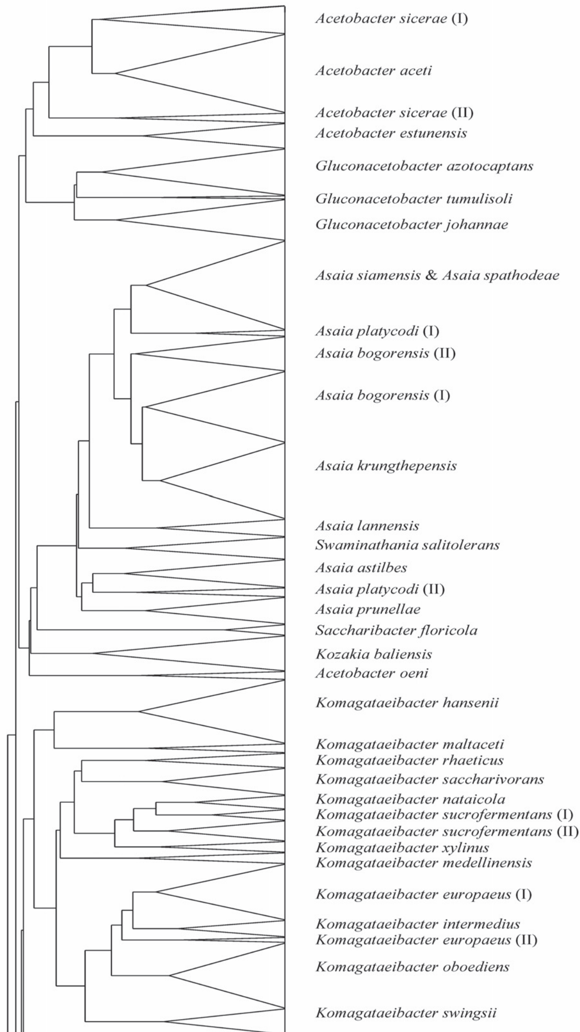
3.3.2. The MALDI-TOF MS AAB identification database

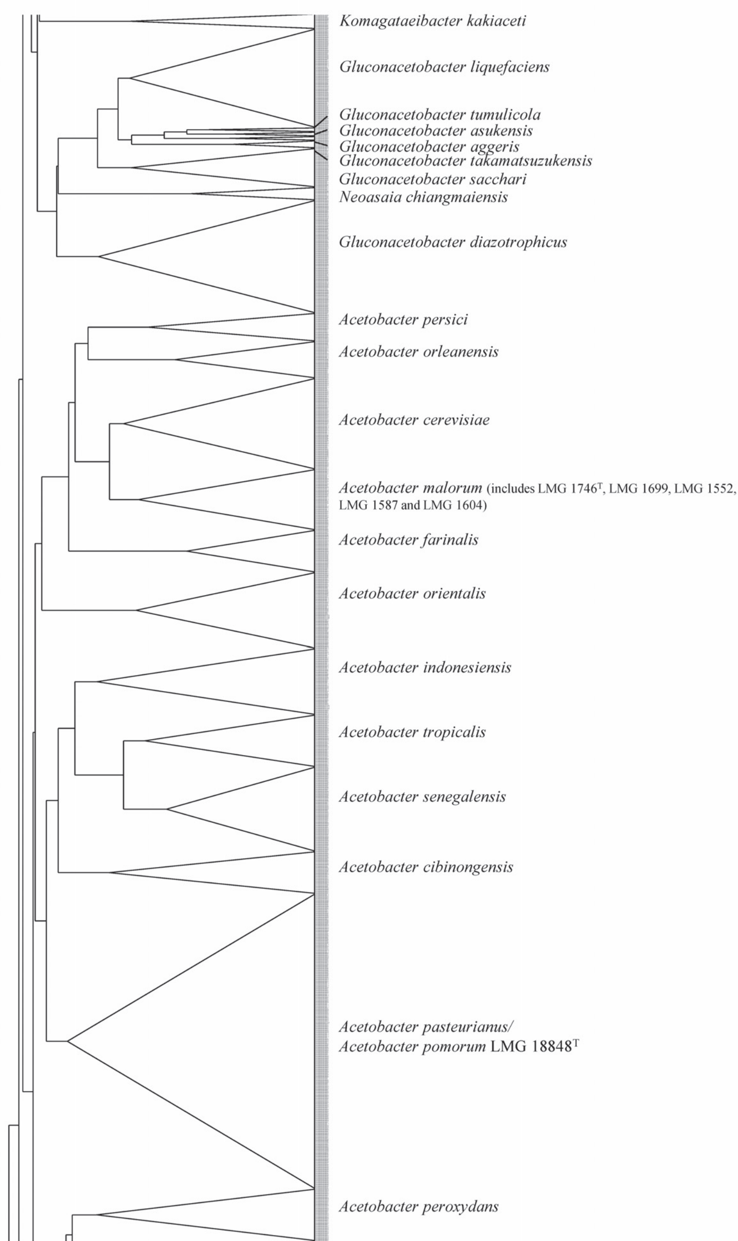
In each dendrogram, most of the strains clustered according to their species designation; however, more species grouped as a single cluster when the Dice coefficient was used. Moreover, a better reflection of the phylogeny was obtained with the Dice coefficient. Indeed, although several *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* species did not cluster with the remaining species belonging to these respective genera, there was a general tendency that most, or all, *Asaia*, *Komagataeibacter*, *Gluconacetobacter*, *Acetobacter* and *Gluconobacter* species grouped into a single genus-specific cluster each; moreover, *Komagataeibacter* and *Gluconacetobacter*, which are phylogenetic nearest neighbors as determined by their 16S rRNA gene divergence (Yamada *et al.*, 2012), grouped together too (Figure 3.1). Therefore, a dendrogram based on profiles analyzed using the Dice coefficient is shown in Figure 3.1 and discussed below.

For *Acetobacter*, *Gluconacetobacter*, *Komagataeibacter* and *Gluconobacter*, most species formed a single cluster. Of the remaining species, *G. japonicus* strains grouped with *G. nephelii* strains, the *A. malorum* type strain grouped with *A. cerevisiae* strains, and the *A. pomorum* type strain grouped with *A. pasteurianus* strains; in addition, spectra of *A. sicerae*, *A. fabarum*, *Kom. europaeus* and *Kom. sucrofermentans* strains split into two clusters each.

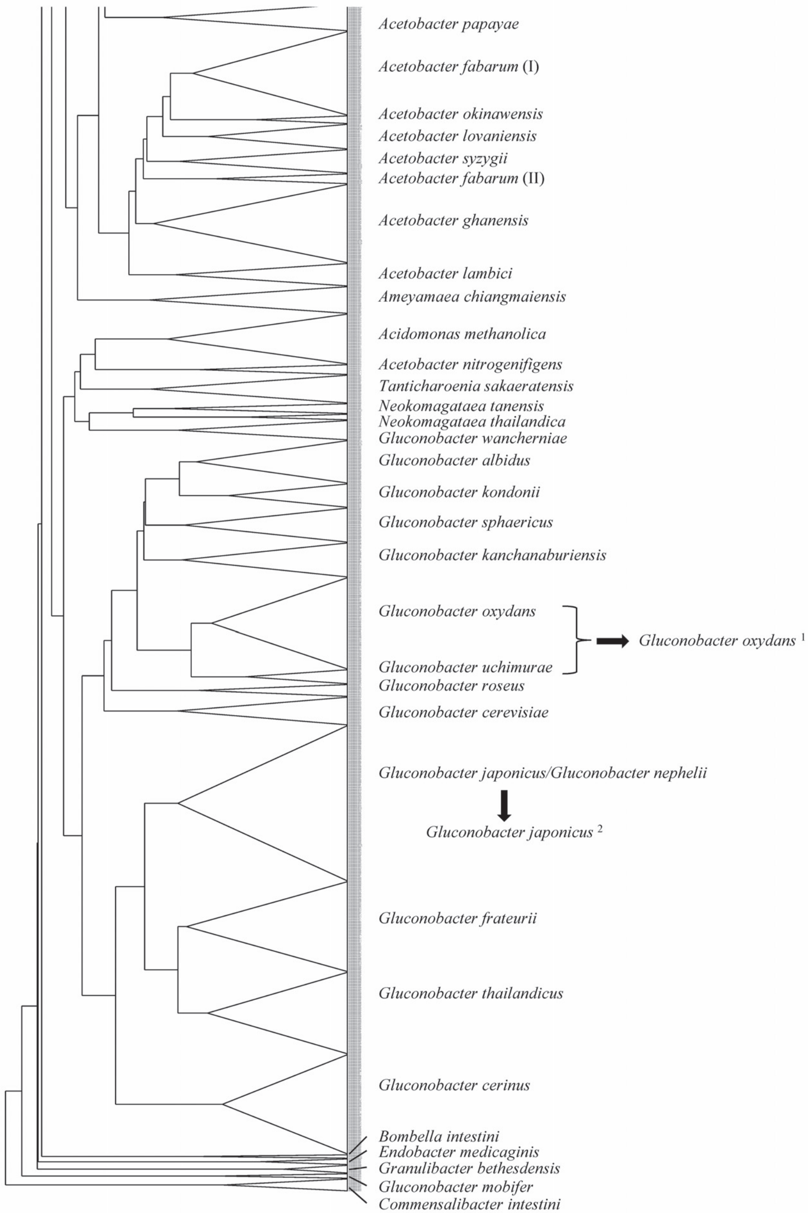
Of *A. malorum* and *A. pomorum*, the type strain of the latter species clustered with strains of *A. pasteurianus*. *A. sicerae* and *A. fabarum*

Figure 3.1. Dendrogram based on MALDI-TOF MS profiles of 279 acetic acid bacteria reference strains. Profile similarities were calculated by the peak-based Dice coefficient. Species that clustered into two subgroups are marked with I and II, of which I contained type strain of the species.





Komagataeibacter kakiaceti
Gluconacetobacter liquefaciens
Gluconacetobacter tumulicola
Gluconacetobacter asukensis
Gluconacetobacter aggeris
Gluconacetobacter takamatsuzukensis
Gluconacetobacter sacchari
Neoasaia chiangmaiensis
Gluconacetobacter diazotrophicus
Acetobacter persici
Acetobacter orleanensis
Acetobacter cerevisiae
Acetobacter malorum (includes LMG 1746T, LMG 1699, LMG 1552, LMG 1587 and LMG 1604)
Acetobacter farinalis
Acetobacter orientalis
Acetobacter indonesiensis
Acetobacter tropicalis
Acetobacter senegalensis
Acetobacter cibinongensis
Acetobacter pasteurianus/
Acetobacter pomorum LMG 18848T
Acetobacter peroxydans


Acetobacter papayae
Acetobacter fabarum (I)
Acetobacter okinawensis
Acetobacter lovaniensis
Acetobacter syzygii
Acetobacter fabarum (II)
Acetobacter ghanensis
Acetobacter lambici
Ameyamaea chiangmaiensis
Acidomonas methanolica
Acetobacter nitrogenifigens
Tanticharoenia sakaeratensis
Neokomagataea tanensis
Neokomagataea thailandica
Gluconobacter wancherniae
Gluconobacter albidus
Gluconobacter kondonii
Gluconobacter sphaericus
Gluconobacter kanchanaburiensis
Gluconobacter oxydans
Gluconobacter oxydans 1
Gluconobacter uchimurae
Gluconobacter roseus
Gluconobacter cerevisiae
Gluconobacter japonicus/Gluconobacter nephelii
Gluconobacter japonicus 2
Gluconobacter frateurii
Gluconobacter thailandicus
Gluconobacter cerinus
Bombella intestini
Endobacter medicaginis
Granulibacter bethesdensis
Gluconobacter mobifer
Commensalibacter intestini
1, strains reclassified as G. oxydans after implementing the ANI data obtained in this study.
2, strains reclassified as G. japonicus after implementing the ANI data obtained in this study.

were subdivided into two subclusters each, mainly due to differences in the number of peaks in the MS profiles. In the case of low signal intensities (< 1500), multiple small peaks were absent, causing these profiles to form a separate cluster. *Kom. europaeus* strain LMG 1380 clustered separately from the other strains of this species. The MS profile of this strain was similar to the profiles of other *Kom. europaeus* strains, except for one major peak that showed a position shift (Figure S3.1). For *Kom. sucrofermentans*, a low number of species-specific peaks in the MS profiles of the strains investigated caused them to cluster into two subclusters. Finally, for *A. pasteurianus*, three strains (LMG 1604, LMG 1587 and LMG 1552) clustered with *A. malorum* LMG 1746^{T} and *A. cerevisiae* LMG 1699, suggesting that they were probably misclassified (Figure S3.2). DNA-DNA hybridizations performed with these strains yielded variable results close to the species delineation threshold (data not shown) making it impossible to identify them to the species level.

Four out of eight *Asaia* species grouped as a separate cluster each, *i.e. As. lannensis*, *As. prunellae*, *As. krunthepensis* and *As. astilbes*. *As. bogorensis* and *As. platycodi* formed two subclusters each, while strains of the phylogenetically closely related species *As. spathodeae* and *As. siamensis* (Kommanee *et al.*, 2010) were intermixed. *As. bogorensis* and *As. platycodi* were subdivided into subclusters due to a low number of species-specific peaks in the MS profiles of the strains investigated.

Spectra of strains of *Neokomagataea tanensis*, *Neokomagataea thailandica* and of the single-species genera *Ameyamaea chiangmaiensis*, *Tanticharoenia sakaeratensis*, *Saccharibacter floricola*, *Swaminathania salitolerans*, *Kozakia baliensis*, *Neoasaia chiangmaiensis*, *Acidomonas methanolica*, "*Commensalibacter intestini*", *Bombella intestini*, *Endobacter medicaginis* and *Granulibacter bethesdensis* grouped in a single cluster each.

3.3.3. Identification of cocoa bean isolates

Thirty out of 33 cocoa bean fermentation isolates (Table 3.1) were identified as *Acetobacter persici*, *Acetobacter okinawensis*, *G. japoni-*

cus/G. nephelii, *G. oxydans/G. uchimurae*, *A. fabarum*, *A. tropicalis*, *A. cerevisiae/A. malorum*, or *A. peroxydans*. The identification of these isolates was confirmed using AFLP, MLSA or DNA-DNA hybridizations (Table 3.1, Figure S3.3, Figure 3.2 and Table S3.3). An interesting finding during the present analysis was that 64 % to 69 % DNA-DNA relatedness was obtained between strains of the recently described species *G. nephelii* and strains of *G. japonicus*. These results were supported by the phylogeny of the strains based on 16S-23S ITS sequences (Kommanee *et al.*, 2011) and housekeeping gene sequences (Figure 3.2), and by AFLP (Figure S3.3) and MALDI-TOF MS data (Figure 3.1). Therefore, all these results correlated well and were in conflict with the low DNA-DNA relatedness values of 34 % to 42 % reported elsewhere (Kommanee *et al.*, 2011). The three remaining cocoa bean isolates formed a very distinct cluster and were identified as *Tatumella* sp., based on 16S rRNA gene sequence analysis (data not shown).

3.3.4. Identification of the BCCM/LMG Bacteria Collection strains

About 65 % of the (presumed) AAB strains obtained from the BCCM/LMG Bacteria Collection could easily be identified to the species level or as belonging to the *G. japonicus/G. nephelii* or *A. cerevisiae/A. malorum* species clusters (Table S3.2), because their spectra consistently clustered among spectra of reference strains of these species. The strains that could not be identified grouped at the border of the *G. albidus*, *G. thailandicus*, *G. oxydans* or *G. uchimurae* clusters, or formed clusters well separated from those of the established species. A representative selection of the strains that formed well separated clusters were investigated through 16S rRNA or housekeeping gene (*dnaK*, *groEL* or *rpoB*) sequencing, which revealed that they were either not AAB, or represented novel AAB species (see below) (Tables S3.2 and S3.4).

Table 3.1. List of isolates of cocoa bean fermentation. MALDI-TOF MS identifications. MALDI-TOF MS results were confirmed by AFLP, MLSA or DNA-DNA hybridization (DDH) data.

Strain number	Original number	Year of isolation	Origin	Sample source [+]	MALDI-TOF MS identification	AFLP identification	MLSA identification	DDH identification	Identified as
R-49105	A22	2007	Brazil	Box2, S0	*A. persici*	*A. persici*	*A. persici*	*A. persici*	*A. persici*
R-49106	62a	2007	Brazil	Box1, Box	*A. persici*				*A. persici*
R-49108	A44b	2007	Brazil	Box 2, S2	*A. persici*				*A. persici*
R-49109	42	2007	Brazil	Box 2, Sack	*A. persici*	*A. persici*	*A. persici*	*A. persici*	*A. persici*
R-49110	53	2007	Brazil	Box 1, Sack	*A. persici*	*A. persici*	*A. persici*		*A. persici*
R-49112	A1051	2008	Ecuador	FINCA 2, S1	*A. persici*				*A. persici*
R-49113	A1066	2008	Ecuador	FINCA 2, S4	*A. persici*	*A. persici*	*A. persici*		*A. persici*
R-49116	A770	2008	Ecuador	FINCA 1, S0	*A. persici*				*A. persici*
R-49127	D451	2008	Ecuador	FINCA 1, Open pod	*A. persici*	*A. persici*	*A. persici*		*A. persici*
R-49130	D900	2008	Ecuador	FINCA 2, S3	*A. persici*	*A. persici*	*A. persici*		*A. persici*
R-49131	D1179	2010	Malaysia	MALAYSIA 2, S1	*A. persici*	*A. persici*	*A. persici*		*A. persici*
R-49111	A1005	2008	Ecuador	FINCA 2, S0	*A. okinawensis*	*A. okinawensis*	*A. okinawensis*		*A. okinawensis*
R-49118	A750	2008	Ecuador	FINCA 1, Open pod	*A. okinawensis*		*A. okinawensis*		*A. okinawensis*
R-49119	A1065	2008	Ecuador	FINCA 2, S4	*A. okinawensis*	*A. okinawensis*	*A. okinawensis*	*A. okinawensis*	*A. okinawensis*
R-49136	D1142	2010	Malaysia	MALAYSIA 1, S0	*A. okinawensis*	*A. okinawensis*	*A. okinawensis*		*A. okinawensis*
R-49114	A824	2008	Ecuador	INIAP 1, S0	*G. japonicus/G. nephelii*	*G. nephelii*	*G. japonicus/G. nephelii*		*G. japonicus* *
R-49115	A723	2008	Ecuador	FINCA 1, Hand	*G. japonicus/G. nephelii*	*G. nephelii*			*G. japonicus* *
R-49117 [#]	A813	2008	Ecuador	INIAP 1, S0	*G. japonicus/G. nephelii*	*G. nephelii*	*G. japonicus/G. nephelii*	*G. japonicus/G. nephelii*	*G. japonicus* *
R-49123	D460	2008	Ecuador	FINCA 1, Open pod	*G. japonicus/G. nephelii*	*G. nephelii*	*G. japonicus/G. nephelii*		*G. japonicus* *
R-49120	A966	2008	Ecuador	INIAP 2, Box	*G. oxydans/G. uchimurae*	*G. oxydans/G. uchimurae*			*G. oxydans* *
R-49121	A1028	2008	Ecuador	INIAP2, S0	*G. oxydans/G. uchimurae*	*G. oxydans/G. uchimurae*			*G. oxydans* *
R-49107	48	2007	Brazil	Box 2, Sack	*A. fabarum*	*A. fabarum*	*A. fabarum*		*A. fabarum*
R-49126	D458	2008	Ecuador	FINCA 1, Open pod	*A. fabarum*	*A. fabarum*	*A. fabarum*		*A. fabarum*
R-49135	D1133	2010	Malaysia	MALAYSIA 1, S0	*A. fabarum*	*A. fabarum*	*A. fabarum*		*A. fabarum*
R-49132	D1151	2010	Malaysia	MALAYSIA 2, S0	*A. tropicalis*	*A. tropicalis*	*A. tropicalis/A. senegalensis*		*A. tropicalis*
R-49137	D1197	2010	Malaysia	MALAYSIA 1, S2	*A. cerevisiae/A. malorum*		*A. cerevisiae/A. malorum*		*A. malorum* *
R-49133	D1186	2010	Malaysia	MALAYSIA 2, S1	*A. peroxydans*	*A. peroxydans*	*A. peroxydans*		*A. peroxydans*
R-50323	D1135	2010	Malaysia	MALAYSIA 1, S0	*A. peroxydans*	*A. peroxydans*			*A. peroxydans*
R-50234	D1155	2010	Malaysia	MALAYSIA 2, S0	*A. peroxydans*	*A. peroxydans*			*A. peroxydans*
R-50325	D1184	2010	Malaysia	MALAYSIA 2, S1	*A. peroxydans*	*A. peroxydans*			*A. peroxydans*
R-49122	D501	2008	Ecuador	INIAP 1, S2	Unidentified				*Tatumella* sp. [$]
R-49124	D493	2009	Ecuador	INIAP 1, S2	Unidentified				*Tatumella* sp. [$]
R-49129	D853	2008	Ecuador	INIAP 2, S2	Unidentified				*Tatumella* sp. [$]

* Taking into account ANI values obtained in this study.

[$] Based on 16S rRNA gene sequencing data.

[#] Strain selected in this study for WGS.

[+] Samples from Brazil: two fermentations were carried out using high-quality organic cocoa from the well maintained 'Hawaii' (Box 1 fermentation) and 'Leão De Ouro' (Box 2 fermentation) plantations; sample numbers (indicated as S) correspond with the time points samples were taken: after 0 (S0), 6 (S1) and 12 (S2) hours of fermentation.

Samples from Ecuador: four fermentations were carried out: one box fermentation in the institute INIAP (INIAP 1), one platform fermentation on the farm (FINCA 1) with cocoa pods from farm 1 (FINCA 1), one box fermentation in the institute INIAP (INIAP 2) and one platform fermentation on the farm (FINCA 2) with cocoa pods from farm 2 (FINCA 2); sample numbers correspond with the time points samples were taken: after 0 (S0), 6 (S1), 12 (S2), 18 (S3) and 24 (S4) hours of fermentation.

Samples from Malaysia: two box fermentations were carried out: MALAYSIA 1 and MALAYSIA 2; sample numbers correspond with the time points samples were taken: after 0 (S0), 6 (S1) and 12 (S2) hours of fermentation.

Swab samples corresponding to a surface of 25 cm^2 were taken from the environment: pod surfaces (Open pod), workers' hands (Hand), transport and fermentation boxes (Box) and sacks (Sack).

3.3.5. MLSA analysis

A representative selection of the unidentified AAB grouping near or among *Gluconobacter* species based on MALDI-TOF MS data (see section 3.3.4), were investigated through MLSA analysis. The sequences were compared with sequences of *Gluconobacter* reference strains (Spitaels *et al.*, 2014a), including those of the cocoa bean isolates R-49114, R-49117 and R-49132. Trees based on individual (data not shown) as well as on concatenated sequences (Figure 3.2) again clustered these strains near or among the same species, as found with MALDI-TOF MS analysis. Three strains (LMG 1406, LMG 1494 and LMG 1680) consistently clustered close to the type strain of *G. oxydans* (99-100 % pairwise sequence similarity for all three housekeeping gene sequences) and were therefore identified as such; the other strains remained unidentified.

3.3.6. Whole-genome sequencing and ANI calculation

To clarify the taxonomic status of the remaining unidentified *Gluconobacter* strains and to resolve some remaining taxonomic ambiguities, 27 strains were selected for WGS. These included (the type) strains of *G. japonicus*, *G. nephelii*, *A. tropicalis*, *A. senegalensis*, *A. cerevisiae* and *A. malorum*; *A. pasteurianus* LMG 1552 and LMG 1604, *G. oxydans* LMG 1406, the cocoa bean isolate R-49117, and representatives of the *Gluconobacter* sp. strains, *i.e.* LMG 1399, LMG 1676, LMG 1386, LMG 1764, LMG 1768 and LMG 1741 (Table 3.2). WGS yielded a minimum of 1,300,321 and a maximum of 3,087,128 reads per strain. Contigs assembled in the CLC Genomics Workbench were submitted to NCBI under BioProject PRJNA288385. The accession numbers and main characteristics of the assembled draft genomes are provided in Table S3.5. Contigs longer than 1000 bp were extracted from each genome and used for ANI calculations, which also included the publicly available *G. oxydans* 621H (GCA_000011685.1) and *A. malorum* DmCS 005 draft genome (GCA_000743885.1) sequences. The ANI values obtained are shown in Table 3.2. Values $>$ 95 %, commonly recommended as ANI threshold for taxonomically circumscribing prokaryotic species (Goris

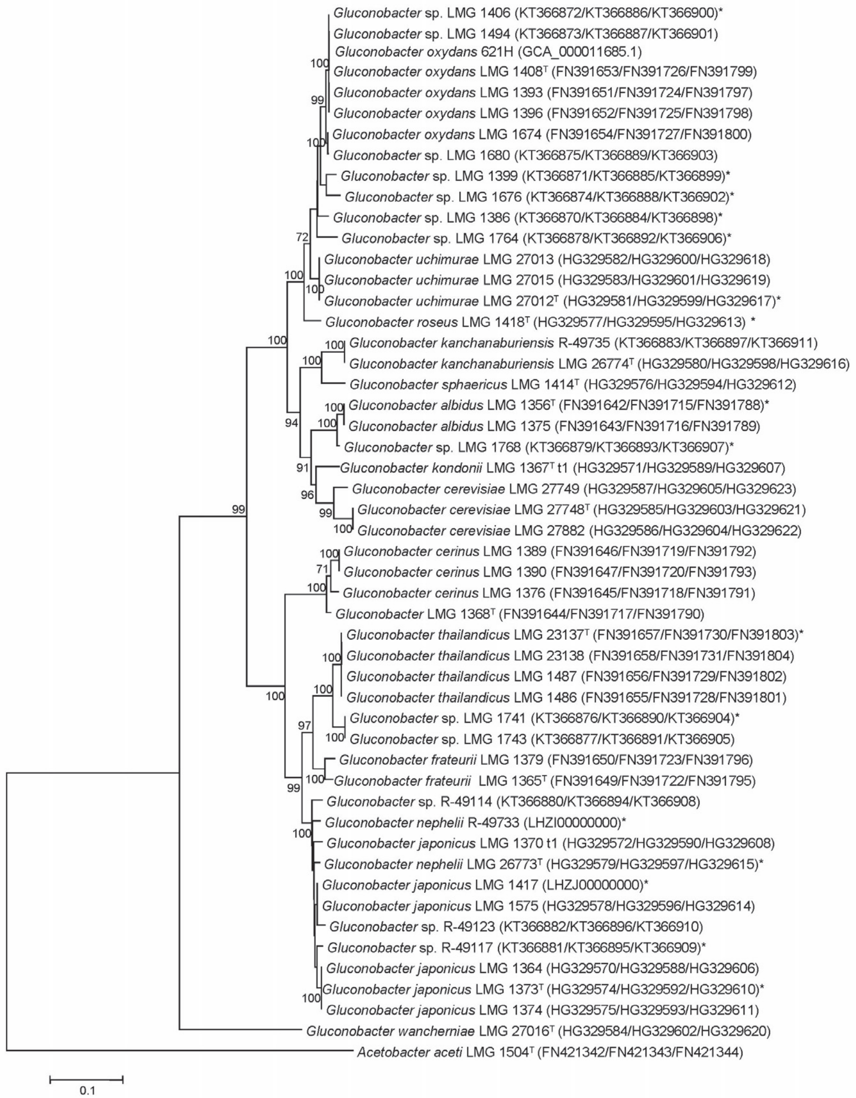


Figure 3.2. Maximum Likelihood tree based on concatenated sequences (1578 bp) of 3 housekeeping gene fragments [*dnaK* (532 bp), *groEL* (534 bp) and *rpoB* (512 bp)] showing the phylogenetic position of species within the genus *Gluconobacter*. The type strain of *Acetobacter aceti* was used as outgroup. Numbers at branching points are percentage bootstrap values based on 1000 replications. The sequence accession numbers for *dnaK*, *groEL* and *rpoB* gene sequences are given between brackets. Bar, 10 % sequence divergence. *, strain selected for WGS.

et al., 2007; Richter and Rossello-Mora, 2009), were found between (1) LMG 1552, LMG 1604, *A. cerevisiae* LMG 1699, and two *A. malorum* strains, including the type strain; (2) LMG 1768 and *G. albidus* LMG 1356^{T}; (3) LMG 1741 and *G. thailandicus* LMG 23137^{T}; (4) R-49117 and two strains each of *G. nephelii* and *G. japonicus*, including both type strains; (5) the *Gluconobacter* sp. strains LMG 1399, LMG 1676, LMG 1386, *G. uchimurae* LMG 27012^{T}, and two *G. oxydans* strains LMG 1406 and 621H; *Gluconobacter* sp. strain LMG 1764 showed an ANI value of 0.944 to 0.948 towards the latter strains.

3.3.7. Phenotypic analysis of *G. japonicus/G. nephelii* and *G. oxydans/G. uchimurae* strains

Phenotypic characteristics reported as useful for the differentiation of *G. nephelii* from *G. japonicus*, *i.e.* acid production from melibiose and L-sorbose, and growth on maltose as the sole carbon source (Kommanee *et al.*, 2011), were re-investigated for 10 strains characterized by MLSA (Figure 3.2), AFLP (Figure S3.3) and/or MALDI-TOF MS (data not shown). However, during the present study, both species could not be distinguished by these features (Table S3.6).

Phenotypic features reported as useful for the differentiation of *G. uchimurae* from the type strain of *G. oxydans*, *i.e.* water-soluble brown pigment, 2,5 diketo-D-gluconic acid production and acid production from maltose (Tanasupawat *et al.*, 2011a) were re-investigated for 9 strains characterized by MLSA (Figure 3.2), AFLP (Figure S3.3) and/or MALDI-TOF MS (data not shown). The results again revealed that these tests were unfit for differentiation of both species (Table S3.7).

3.4. Discussion

AAB are involved in various food processes, and are of interest to the biotechnology industry because of their capacity to produce cellulose or oxidize different kinds of carbohydrates into products of commercial value (Raspor and Goranovič, 2008). In addition, they have been found as symbionts of different insects and as opportunistic human pathogens

Table 3.2. Average Nucleotide Identity (ANI) values among (draft) whole genome sequences of selected AAB strains. Strains with ANI values above 95 % are considered to belong to the same species. MuMmer algorithms was used for the alignment of sequences.

Species	Strain	**LMG 1625^T**	LMG 1545	LMG 1608	LMG 1699	LMG 1604	LMG 1552	**LMG 1746^T**	DmCS_005 $	**LMG 1583^T**	LMG 19825	LMG 1663	**LMG 23690^T**
A. cerevisiae	**LMG 1625^T**	NA											
A. cerevisiae	LMG 1545	0.966	NA										
A. cerevisiae	LMG 1608	0.966	0.971	NA									
A. cerevisiae	LMG 1699	0.934	0.928	0.930	NA								
A. pasteurianus	LMG 1604	0.934	0.929	0.931	0.966	NA							
A. pasteurianus	LMG 1552	0.934	0.929	0.930	0.959	0.958	NA						
A. malorum	**LMG 1746^T**	0.933	0.927	0.928	0.961	0.961	0.960	NA					
A. malorum	DmCS_005 $	0.940	0.929	0.929	0.960	0.961	0.961	0.964	NA				
A. orleanensis	**LMG 1583^T**	0.863	0.862	0.864	0.862	0.863	0.863	0.863	0.866	NA			
A. tropicalis	**LMG 19825^T**	0.855	0.856	0.854	0.853	0.852	0.858	0.842	0.847	0.843	NA		
A. tropicalis	LMG 1663	0.857	0.855	0.856	0.850	0.868	0.862	0.844	0.852	0.845	0.979	NA	
A. senegalensis	**LMG 23690^T**	0.852	0.848	0.850	0.844	0.842	0.847	0.848	0.857	0.842	0.930	0.930	NA

$ Sequence taken from NCBI

Received as *Acetobacter tropicalis* but identified as *Acetobacter senegalensis* based on AFLP data obtained in this study.

Species	Strain	621H	LMG 1406	LMG 1399	LMG 1676	LMG 1386	**LMG 27012^T**	LMG 1764	**LMG 1418^T**	**LMG 1356^T**	LMG 1768	**LMG 23137^T**	LMG 1741	**LMG 1373^T**	R-49117	LMG 1417	R-49733	**LMG 26773T^T**
G. oxydans	621H $	NA																
G. oxydans+	LMG 1406	0.998	NA															
*G. oxydans**	LMG 1399	0.977	0.977	NA														
*G. oxydans**	LMG 1676	0.963	0.965	0.961	NA													
*G. oxydans**	LMG 1386	0.962	0.962	0.963	0.959	NA												
G. uchimurae	**LMG 27012^T**	0.962	0.964	0.960	0.959	0.962	NA											
G. oxydans+	LMG 1764	0.946	0.946	0.946	0.944	0.948	0.945	NA										
G. roseus	**LMG 1418^T**	0.900	0.900	0.900	0.901	0.901	0.901	0.896	NA									
G. albidus	**LMG 1356^T**	0.869	0.870	0.869	0.873	0.869	0.870	0.870	0.866	NA								
*G. albidus**	LMG 1768	0.872	0.873	0.871	0.876	0.869	0.871	0.874	0.868	0.977	NA							
G. thailandicus	**LMG 23137^T**	0.842	0.843	0.848	0.842	0.841	0.840	0.844	0.838	0.844	0.849	NA						
*G. thailandicus**	LMG 1741	0.857	0.849	0.845	0.852	0.844	0.846	0.851	0.837	0.852	0.862	0.974	NA					
G. japonicus	**LMG 1373^T**	0.847	0.851	0.849	0.851	0.839	0.842	0.851	0.839	0.848	0.858	0.873	0.875	NA				
G. japonicus/G. nephelii	R-49117	0.844	0.845	0.844	0.843	0.838	0.841	0.847	0.838	0.846	0.855	0.874	0.876	0.960	NA			
G. japonicus	LMG 1417	0.851	0.855	0.854	0.853	0.837	0.842	0.853	0.838	0.842	0.858	0.875	0.873	0.970	0.961	NA		
G. nephelii	R-49733	0.859	0.854	0.851	0.860	0.846	0.853	0.860	0.842	0.858	0.864	0.878	0.881	0.968	0.960	0.968	NA	
G. nephelii	**LMG 26773T^T**	0.852	0.846	0.846	0.850	0.841	0.843	0.851	0.838	0.850	0.856	0.874	0.876	0.968	0.960	0.968	0.973	NA

+ Strain identified as *G. oxydans* based on ANI data in combination with MLSA and phenotypic data obtained in this study.

* *Gluconobacter* sp. based on MALDI-TOF MS data

$ Sequence taken from NCBI

(Trček and Barja, 2014). A method that enables a quick and accurate identification of AAB would be convenient to study the processes in which these microorganisms are involved. In the present study, MALDI-TOF MS was evaluated for the fast and accurate identification of AAB. Reference strains that formed intermixed clusters after numerical analysis were further investigated, except for strains of *As. spathodeae* and *As. siamensis*, which are phylogenetically closely related species, and *A. pomorum*, which grouped with *A. pasteurianus* strains. The latter species is known to be genotypically diverse (Cleenwerck *et al.*, 2009) and a reassessment of the taxonomy of *A. pomorum* will require a detailed analysis of a large number of *A. pasteurianus* strains as well. Numerical analysis of spectra by means of the peak-based Dice coefficient not only yielded the best species level differentiation, but also best reflected the phylogeny of many species of the family *Acetobacteraceae*. The analysis of spectra generated from cells grown on different cultivation media revealed that species level identification results were generally not affected by the growth medium used, confirming previous data (Wieme *et al.*, 2014a). Only when suboptimal growth was found, the quality of the profiles was more often insufficient for accurate identification, again confirming previous reports (Robbins *et al.*, 2007).

Of the nearly 300 strains examined for evaluation of the database, more than 90 % could be accurately identified to the species level, after implementing the reclassifications as revealed by subsequent taxonomic studies carried out during the present study. A few of these strains (LMG 1383, LMG 1407, LMG 1523, LMG 1549, LMG 1597, LMG 1607, LMG 1678, LMG 1698 and LMG 1743) were investigated previously by MALDI-TOF MS (Andrés-Barrao *et al.*, 2013; Wieme *et al.*, 2014a) and our results confirmed or supported the results from those studies. Overall, the identifications from the nearly 300 strains revealed a large diversity among the BCCM/LMG strains investigated, with several ones belonging to recently described species, such as *A. okinawensis*, *A. sicerae*, *G. takamatsuzukensis*, *G. japonicus* or *Kom. medellinensis*, thus providing new information on the occurrences of these species. Of the isolates from cocoa bean fermentations carried out in Ecuador, Brazil and Malaysia in the period 2007-2010, approximately half were

assigned to the recently described species *A. persici*, *A. okinawensis* or *G. japonicus*, species first reported in 2009 and 2012 (Iino *et al.*, 2012a; Malimas *et al.*, 2009b). These species were never isolated from cocoa bean fermentation samples and environments before. Common *Acetobacter* species involved in cocoa bean fermentation processes are *A. pasteurianus* and *A. tropicalis* (Crafack *et al.*, 2013; Illeghems *et al.*, 2013b; Moens *et al.*, 2014; Papalexandratou *et al.*, 2013). Of the 23 strains that could not be identified to the species level, ten formed clusters well-separated from all established AAB species and subsequent sequence analyses revealed that they were no AAB: seven were members of *Tatumella* (Table S3.2), while strains LMG 1353, LMG 1752 and LMG 1757 were members of the genera *Pantoea*, *Enterococcus* and *Rosenbergiella*, respectively (Table S3.4). Furthermore, strain LMG 1586 was identified as *A. oeni*. The latter species was represented only by the type strain (LMG 21952^{T}) in our reference database and the LMG 1586 spectra clustered at the border of the *A. oeni* LMG 21952^{T} cluster. Its identification failure was likely due to an insufficient number of reference strains of this species. In addition, three strains (LMG 1556, LMG 1620 and LMG 1685 t2) were identified as *A. pasteurianus*. A larger selection of *A. pasteurianus* strains, representing the presently known genotypic diversity in the species, should likely be present in the reference database to reliably identify members of this species. Finally, the remaining 9 strains formed 6 separate clusters among the reference AAB species and multilocus or 16S rRNA gene sequence analyses suggested they may represent 6 novel species of the family *Acetobacteraceae* (Table S3.4).

DNA-DNA hybridization data have long been considered the gold standard to delineate bacterial species (Wayne *et al.*, 1987). However, this type of experimental work is time-consuming, not always reliable, and sometimes provides contradictory results, especially when the values obtained are close to the species delineation threshold [Table S3.3 and (Kommanee *et al.*, 2011)]. In the last decade, MLSA and calculation of ANI values have been proposed as alternative methods for DNA-DNA hybridization experiments (Gevers *et al.*, 2005; Konstantinidis and Tiedje, 2005; Vandamme and Peeters, 2014). In the present study,

ANI values revealed that *A. malorum* and *A. cerevisiae* are genuinely distinct species, with the misclassified strains LMG 1699, LMG 1552, LMG 1587 and LMG 1604 belonging to the former, whereby the misclassification of the latter two strains has been reported in a previous study (Papalexandratou *et al.*, 2009; Wieme *et al.*, 2014a). These data also revealed that MALDI-TOF MS effectively allowed to distinguish among these two closely related species. Growth on methanol as the sole carbon source and growth on yeast extract + 30 % D-glucose failed to differentiate the misclassified strains from the latter species. Hence these phenotypic characteristics are not useful for the differentiation of *A. malorum* and *A. cerevisiae*. For the *G. japonicus*/*G. nephelii* species cluster, MLSA data based on partial sequences of the housekeeping genes *dnaK*, *groEL* and *rpoB* failed to differentiate these two species, and ANI values confirmed that *G. nephelii* and *G. japonicus* represented a single genospecies. Finally, although *G. uchimurae* and *G. oxydans* reference strains occupied distinct positions in the concatenated MLSA tree, several other strains occupied intermediate phylogenetic positions (Figure 3.2). Not surprisingly, *G. uchimurae* LMG 27012^{T} showed ANI values higher than 95 % with *G. oxydans* 621H and several *Gluconobacter* sp. strains, indicating that *G. uchimurae* and *G. oxydans* also represent a single species. A single strain, *i.e. Gluconobacter* sp. strain LMG 1764 showed ANI values just below the 95 % ANI threshold (94.4 – 94.8 %) towards *G. oxydans* strains. Yet, based on MLSA (Figure 3.2) and phenotypic data (Table S3.7), this strain could not be differentiated from strains of this species. We therefore propose to classify strain LMG 1764 as *G. oxydans*, as phenotypic and phylogenetic data should be in agreement with the proposed classifications.

Together, not only the phylogenies of *G. nephelii*, *G. japonicus*, *G. uchimurae* and *G. oxydans* based on 16S-23S ITS sequences (Kommanee *et al.*, 2011; Spitaels *et al.*, 2014a) and partial sequences of the housekeeping genes *dnaK*, *groEL* and *rpoB* (Figure 3.2), but also AFLP and MALDI-TOF MS data (Figure 3.1 and Figure S3.3) supported the synonymy of *G. nephelii* and *G. japonicus*, and of *G. uchimurae* and *G. oxydans*. Moreover, phenotypic features reported as useful for differentiation of *G. nephelii* and *G. uchimurae* from *G. japonicus* and

G. oxydans, respectively, proved unreliable. We therefore conclude that *G. nephelii* and *G. uchimurae* should be considered later synonyms of *G. japonicus* and *G. oxydans*, respectively (Lapage *et al.*, 1992). Below, an emended description of *G. japonicus* is given. The description of *G. oxydans* requires no emendation, as the characteristics of *G. uchimurae* [(Tanasupawat *et al.*, 2011a) and Table S3.7] fit the present description of *G. oxydans*.

Emended description of *G. japonicus* Malimas *et al.* 2009 The description of *G. japonicus* is as reported by Malimas *et al.* (2009) (Malimas *et al.*, 2009b), except that results for growth on maltose and acid production from melibiose and L-sorbose are strain-dependent. The type strain is NBRC 3271^T (= BCC 14458^T = LMG 26773^T), isolated from fruit of *Myrica rubra*.

3.5. Acknowledgements

The BCCM/LMG collection is supported by the Federal Public Planning Service-Science Policy, Belgium. The authors acknowledge the financial support of the Research Foundation-Flanders (FWO-Vlaanderen), the Research Council of Ghent University and the Vrije Universiteit Brussel (SRP, IRP, and IOF projects), and of the Hercules Foundation. L.L. acknowledges the Chinese Scholarship Council and Ghent University Co-Funding. In addition, the authors would like to acknowledge Zoi Papalexandratou and Timothy Lefeber who carried out the cocoa bean fermentations.

3.6. Supplementary materials

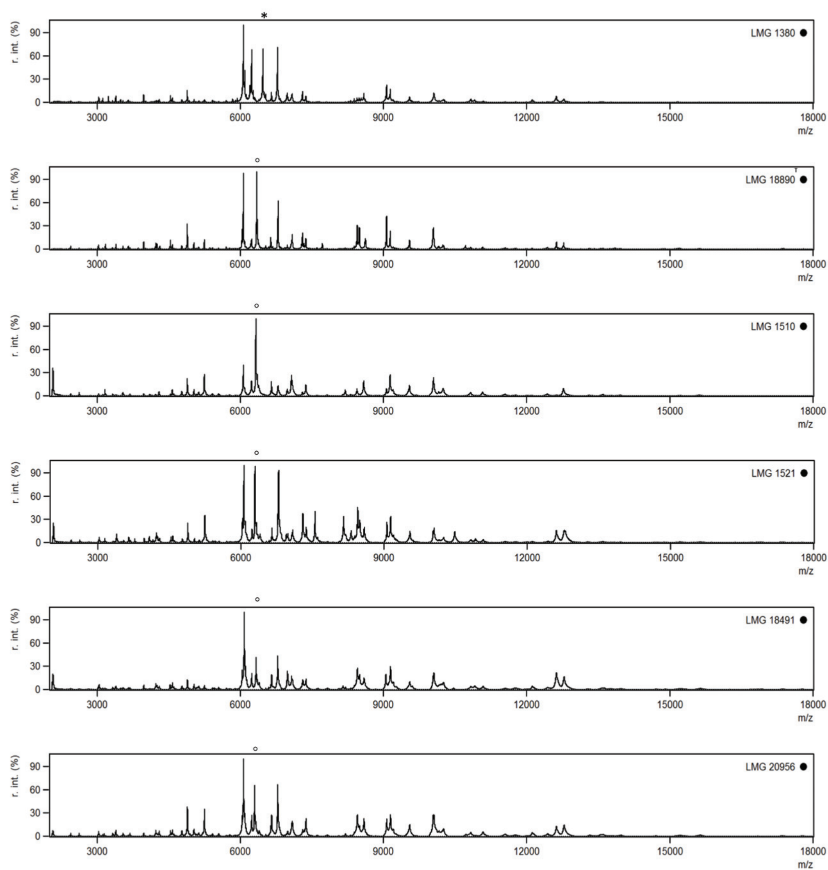


Figure S3.1. MALDI-TOF MS profiles of *Komagataeibacter europaeus* strains. *, peak only present in strain LMG 1380. ○, peak absent in strain LMG 1380.

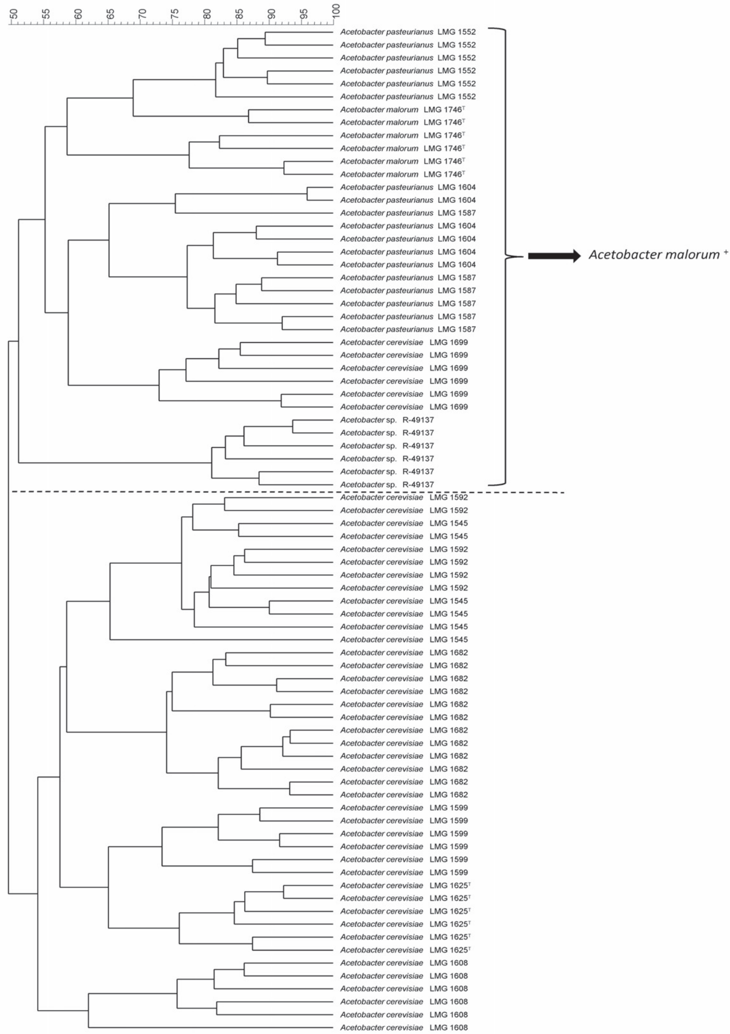


Figure S3.2. UPGMA dendrogram based on MALDI-TOF MS profiles of *Acetobacter pasteurianus* LMG 1604, LMG 1587 and LMG 1552, *Acetobacter* sp. R-49137, strains of *Acetobacter cerevisiae* and the type strain of *Acetobacter malorum*. Similarities were calculated using the peak-based Dice coefficient.

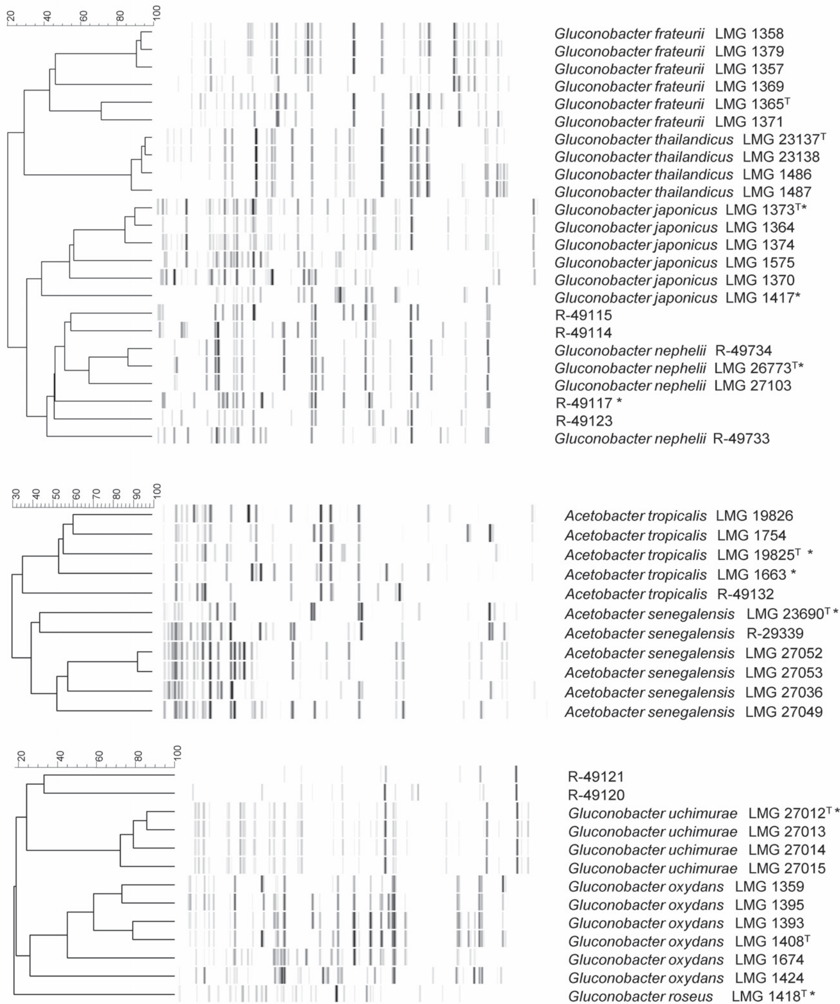


Figure S3.3. AFLP™ DNA fingerprints of (A) *G. japonicus* and *G. nephelii* strains and their closest phylogenetic relatives; (B) *G. oxydans*, *G. uchimurae* and their closest phylogenetic ralative; (C). *A. senegalensis* and *A. tropicalis* strains. The dendrogram was derived from UPGMA of the fingerprints with levels of linkage expressed as Dice similarity coefficients. *, strain selected for WGS.

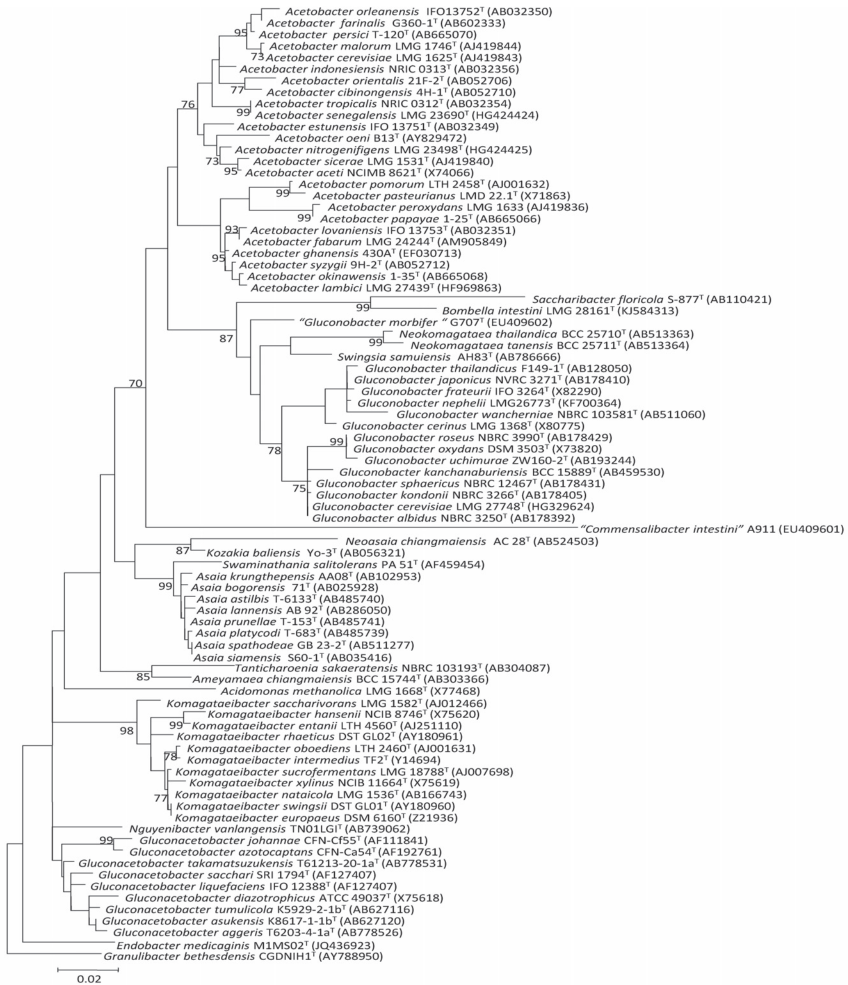


Figure S3.4. Maximum-likelihood tree based on nearly complete 16S rRNA gene sequences (1365 bp) of the type strains of all species of family *Acetobacteraceae* showing the phylogenetic relationships of this family. *Granulibacter bethesdensis* CGDNIH1^{T} was used as outgroup. The phylogenetic tree was constructed using MEGA 6. The DNA substitution K2+G+I was selected. The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets. Bar, 2 % sequence divergence.

Table S3.1. List of AAB strains selected for the construction of a MALDI-TOF MS identification database.

Strain number used in study	Species name	Received as	Geographic origin	Biological origin (Italic)
LMG 1496	*Acetobacter aceti*			
LMG 1504^{T}	***Acetobacter aceti***			Beech-wood shavings of vinegar plant
LMG 1505	*Acetobacter aceti*		Zwolle	Quick vinegar
LMG 1512	*Acetobacter aceti*		Aichi, Japan	Film in fermentor of rice vinegar
LMG 1535	*Acetobacter aceti*		Belgium	Vinegar plant
LMG 18491 t1	*Acetobacter aceti*		Kolinska, Slovenia	Cider vinegar
LMG 1545	*Acetobacter cerevisiae*		Aichi, Japan	Film in fermentor of rice vinegar
LMG 1592	*Acetobacter cerevisiae*			Manufacture of vinegar
LMG 1599	*Acetobacter cerevisiae*		United Kingdom	Brewers' yeast and beer
LMG 1608	*Acetobacter cerevisiae*		The Netherlands	Beer
LMG 1625^{T}	***Acetobacter cerevisiae***		Toronto, Canada	Beer (ale) in storage
LMG 1682	*Acetobacter cerevisiae*		Ireland	Beer
LMG 21418^{T}	***Acetobacter cibinongensis***		Indonesia	*Annona montanae*
LMG 27059 *	*Acetobacter cibinongensis*		Finland	Brewery
LMG 1572	*Acetobacter estunensis*		Bristol, United Kingdom	Cider
LMG 1580	*Acetobacter estunensis*		Leiden, The Netherlands	Beer
LMG 1626^{T}	***Acetobacter estunensis***		Bristol, United Kingdom	Cider
LMG 24244^{T}	***Acetobacter fabarum***		Ghana	Fermented cocoa beans
LMG 24630	*Acetobacter fabarum* $	*Acetobacter lovaniensis*	Gembloux, Belgium	Kefir grains (dairy starter)
LMG 27039 *	*Acetobacter fabarum* $	*Acetobacter lovaniensis*	Central Thailand	Mushroom
LMG 27041 *	*Acetobacter fabarum* $	*Acetobacter lovaniensis*	Central Thailand	Fruit of *Artocarpus heterophyllus*
LMG 27044 *	*Acetobacter fabarum* $	*Acetobacter lovaniensis*	Central Thailand	Flower of *Heliconia* sp.
R-36331	*Acetobacter fabarum*		Ghana	Fermented cocoa beans
R-36458	*Acetobacter fabarum*		Ghana	Fermented cocoa beans
R-36459	*Acetobacter fabarum*		Ghana	Fermented cocoa beans
LMG 26772^{T}	***Acetobacter farinalis***		Thailand	Fermented starch
LMG 27045 *	*Acetobacter farinalis*		Central Thailand	Fermented starch
LMG 27046 *	*Acetobacter farinalis*		Central Thailand	Fermented starch
LMG 23848^{T}	***Acetobacter ghanensis***		Ghana	Fermented cocoa beans
LMG 27093 *	*Acetobacter ghanensis*		Pathumthani, Thailand	Peach
R-29336	*Acetobacter ghanensis*		Ghana	Fermented cocoa beans
R-29338	*Acetobacter ghanensis*		Ghana	Fermented cocoa beans
LMG 1571	*Acetobacter indonesiensis*			
LMG 1588	*Acetobacter indonesiensis*			
LMG 19824^{T}	***Acetobacter indonesiensis***		Indonesia	Fruit of zirzak (*Annona muricata*)
LMG 27037 *	*Acetobacter indonesiensis*		Northern Thailand	Fruit of *Aglaia* ap.
LMG 27096 *	*Acetobacter indonesiensis*		Uttaradit, Thailand	Fermented rice flour
LMG 27439^{T}	***Acetobacter lambici***		Belgium	Fermenting lambic beer (Industrial type brewery)
LMG 27440	*Acetobacter lambici*		Belgium	Fermenting lambic beer (Traditional type brewery)
R-50193	*Acetobacter lambici*		Belgium	Fermenting lambic beer (Traditional type brewery)
R-50194	*Acetobacter lambici*		Belgium	Fermenting lambic beer (Industrial type brewery)
LMG 1617^{T}	***Acetobacter lovaniensis***		Becquevoort, Belgium	Sewage on soil
LMG 27097 *	*Acetobacter lovaniensis*		Uttaradit, Thailand	Fermented rice flour
LMG 1746^{T}	***Acetobacter malorum***		Ghent, Belgium	Rotting apple
LMG 1552	*Acetobacter malorum* #	*Acetobacter pasteurianus*		Probably *Malus* sp.
LMG 1587	*Acetobacter malorum* #	*Acetobacter pasteurianus*		Beer (ale)
LMG 1604	*Acetobacter malorum* #	*Acetobacter pasteurianus*	United Kingdom	Sour beer
LMG 1699	*Acetobacter malorum* #	*Acetobacter cerevisiae*	United Kingdom	Brewery
LMG 23498^{T}	***Acetobacter nitrogenifigens***		India	Kombucha tea
LMG 21952^{T}	***Acetobacter oeni***		Viseu Dao region, Portugal	Spoiled red wine
LMG 26457^{T}	***Acetobacter okinawensis***		Okinawa, Japan	Stem of sugarcane
LMG 1547	*Acetobacter orientalis*		Lockyer Vally Queensland, Austra	Black earth soil
LMG 21417^{T}	***Acetobacter orientalis***		Indonesia	*Canna hybrida*
LMG 27038 *	*Acetobacter orientalis*		Central Thailand	Flower of *Canna* sp.
LMG 27042 *	*Acetobacter orientalis*		Central Thailand	Fruit
LMG 27043 *	*Acetobacter orientalis*		Central Thailand	Fruit of *Artocarpus heterophyllus*
LMG 27098 *	*Acetobacter orientalis*		Pathumthani, Thailand	Loog-pang khaomak
LMG 1583^{T}	***Acetobacter orleanensis***		Belgium	Beer
LMG 27270	*Acetobacter orleanensis*			
LMG 26456^{T}	***Acetobacter papayae***		Okinawa, Japan	Papaya fruit
LMG 27040 *	*Acetobacter papayae* $	*Acetobacter peroxydans*	Central Thailand	Fruit of *Psidium guajava*
LMG 1262^{T}	***Acetobacter pasteurianus***		The Netherlands	Beer
LMG 1553	*Acetobacter pasteurianus*		Osaka, Japan	Spoiled beer

LMG 1555	*Acetobacter pasteurianus*			
LMG 1590	*Acetobacter pasteurianus*			
LMG 1591	*Acetobacter pasteurianus*			Quick vinegar
LMG 1618	*Acetobacter pasteurianus*		United Kingdom	Malt vinegar acetifier
LMG 1629	*Acetobacter pasteurianus*		Recife, Brazil	Fermented *Agave sisalana* juice
LMG 1630	*Acetobacter pasteurianus*		Recife, Brazil	*Saccharum officinarum bagasse*
LMG 1658	*Acetobacter pasteurianus*		Myanmar	
LMG 1659	*Acetobacter pasteurianus*		Myanmar	
LMG 1686	*Acetobacter pasteurianus*		The Netherlands	Vinegar from dry raisins
LMG 27050 *	*Acetobacter pasteurianus* $^{\$}$	*Acetobacter aceti*	Thailand	Fruit
LMG 27051 *	*Acetobacter pasteurianus* $^{\$}$	*Acetobacter aceti*	Thailand	Red wine
LMG 23182	*Acetobacter pasteurianus*		Australia	Oxidized Shiraz wine showing spoilage
R-29340	*Acetobacter pasteurianus*		Ghana	Fermented cocoa beans
R-29341	*Acetobacter pasteurianus*		Ghana	Fermented cocoa beans
R-29342	*Acetobacter pasteurianus*		Ghana	Fermented cocoa beans
LMG 1633	*Acetobacter peroxydans*		Netherlands	Ditch water
LMG 1635^{T}	***Acetobacter peroxydans***		Delft, The Netherlands	Ditch water
LMG 21769	*Acetobacter peroxydans*		Tamilnadu, India	Root tissue of *Oryza sativa*
LMG 21770	*Acetobacter peroxydans*		Tamilnadu, India	Root tissue of wetland rice
LMG 1805	*Acetobacter persici* $^{\$}$	*Acetobacter pasteurianus*	United Kingdom	*Malus sylvestris*
LMG 26458^{T}	***Acetobacter persici***		Tottori Prefecture, Japan	A peach fruit
LMG 27271	*Acetobacter persici*			
LMG 18848^{T}	***Acetobacter pomorum***		Esslingen, Germany	Cider vinegar fermentation
LMG 23690^{T}	***Acetobacter senegalensis***		Casamance, Senegal	Mango fruit
LMG 27036 *	*Acetobacter senegalensis* $^{\$}$	*Acetobacter tropicalis*	Thailand	
LMG 27049 *	*Acetobacter senegalensis* $^{\$}$	*Acetobacter tropicalis*	Thailand	
LMG 27052 *	*Acetobacter senegalensis* $^{\$}$	*Acetobacter tropicalis*	Northern Thailand	Fruit of *Annona squamosa*
LMG 27053 *	*Acetobacter senegalensis* $^{\$}$	*Acetobacter tropicalis*	Thailand	Rose apple
R-29339	*Acetobacter senegalensis*		Ghana	Fermented cocoa beans
R-29343	*Acetobacter senegalensis*		Ghana	Fermented cocoa beans
LMG 1530	*Acetobacter sicerae*			Cider
LMG 1531^{T}	***Acetobacter sicerae***			Celluloseless mutant of LMG 1530 isolated from cid
LMG 27543	*Acetobacter sicerae*		Belgium	Kefir
LMG 28092	*Acetobacter sicerae*		Belgium	Kefir
LMG 21419^{T}	***Acetobacter syzygii***		Indonesia	*Syzyguim malaccense*
LMG 27101 *	*Acetobacter syzygii*		Bangkok, Thailand	*Sapodilla*
LMG 1663	*Acetobacter tropicalis*		United Kingdom	Fermenting putrified meat sample
LMG 1754	*Acetobacter tropicalis*		Ivory Coast	Fruit of Ficus capensis
LMG 19825^{T}	***Acetobacter tropicalis***		Indonesia	Coconut (*Coccos nucifera*)
LMG 19826	*Acetobacter tropicalis*		Indonesia	Lime
LMG 1667	*Acidomonas methanolica*			Sludge
LMG 1668^{T}	***Acidomonas methanolica***		Leipzig, Germany	Fermentation of methanol with *Candida* sp. (not ster
LMG 1669	*Acidomonas methanolica*			Sludge
LMG 1735	*Acidomonas methanolica*		Australia	Surface microflora of *Saccharum officinarum*
LMG 27010^{T} *	***Ameyamaea chiangmaiensis***		Chaing Mai, Thailand	Flower of red ginger (*Alpinia purpurea*)
NBRC 103197 *	*Ameyamaea chiangmaiensis*		Chaing Mai, Thailand	Flower of red ginger (*Alpinia purpurea*)
LMG 26974^{T} *	***Asaia astilbes***		Japan, Yamanashi Prefecture	*Astilbe thunbergii var.congesta*
LMG 27005 *	*Asaia astilbes*		Niigata, Japan	Manyspiny knotweed (*Persicaria senticosa*)
LMG 27006 *	*Asaia astilbes*		Niigata, Japan	Asian dayflower (*Commelina communis*)
LMG 21650^{T}	***Asaia bogorensis***		Bogor, Indonesia	Flower of orchid tree (*Bauhinia purpurea*)
LMG 23141	*Asaia bogorensis*		Bogor, Indonesia	Flower of orchid tree (*Bauhinia purpurea*)
LMG 23142	*Asaia bogorensis*		Bogor, Indonesia	Flower of *Plumbago auriculata*
LMG 23143	*Asaia bogorensis*		Yogyakarta, Indonesia	Tape kefan (fermented glutinous rice)
LMG 27034 *	*Asaia bogorensis*		Central Thailand	Flower of *Citharexylum spinosum*
LMG 27285 *	*Asaia bogorensis*		Chiang Mai, Thailand	Flower of *Allamanda cathatica*
NBRC 103412*	*Asaia bogorensis*		Bangkok Thailand	Flower of *Hibiscus* sp.
NBRC 103479 *	*Asaia bogorensis*		Thong Pha Phum,Thailand	Flower of *Ipomoea* sp.
LMG 23083^{T}	***Asaia krungthepensis***		Bangkok, Thailand	Flower of *Heliconia* sp.
LMG 23139	*Asaia krungthepensis*		Bangkok, Thailand	Flower of *Heliconia* sp.
LMG 23140	*Asaia krungthepensis*		Bangkok, Thailand	Flower of *Heliconia* sp.
LMG 27030 *	*Asaia krungthepensis*		Central Thailand	Flower of *Heliconia* sp.
LMG 27033 *	*Asaia krungthepensis*		Central Thailand	Flower of *Heliconia* sp.
LMG 27283 *	*Asaia krungthepensis*		Nonthaburi, Thailand	Fruit of *Coccinia grandis*
NBRC 103419 *	*Asaia krungthepensis*		Bang Bua Tong, Thailand	Fruit of *Sandoricum indicum*
LMG 27011^{T} *	***Asaia lannensis***		Chiang Mai, Thailand	Flower of spider lily (*Crinum asiaticum*)
NBRC 102527 *	*Asaia lannensis*		Chiang Mai, Thailand	Fower of spider lily (*Crinum asiaticum*)
LMG 26975^{T} *	***Asaia platycodi***		Japan, Akita	Balloon flower of *Platycodon grandiflorum*

LMG 27002 *	*Asaia platycodi*		Tokyo, Japan	Unidentified flower
LMG 26976^{T} *	***Asaia prunellae***		Japan, Akita	Self-heal (*Prunella vulgaris*)
LMG 27003 *	*Asaia prunellae*		Akita, Japan	Self-heal (*Prunella vulgaris*)
LMG 27004 *	*Asaia prunellae*		Akita, Japan	Unidentified flower
LMG 21651^{T}	***Asaia siamensis***		Bangkok, Thailand	Crown flower (*Calotropis gigantea*)
LMG 23136	*Asaia siamensis*		Bogor, Indonesia	Flower of spider lily (*Crinum asiaticum*)
LMG 23144	*Asaia siamensis*		Bogor, Indonesia	Flower of spider lily (*Crinum asiaticum*)
LMG 23145	*Asaia siamensis*		Bogor, Indonesia	Flower of spider lily (*Crinum asiaticum*)
LMG 23146	*Asaia siamensis*		Bogor, Indonesia	Flower of *Ixora chinensis*
LMG 27284 *	*Asaia siamensis*		Northern Thailand	Flower of *Ixora* sp.
LMG 27047^{T} *	***Asaia spathodeae***		Thailand	Flower of *Spathodea companulata*
LMG 27288 *	*Asaia spathodeae*		Thailand	Flower of *Spathodea companulata*
NBRC 103415 *	*Asaia spathodeae* $	*Asaia siamensis*	Samutsakorn, Thailand	Fruit of pomelo (*Citrus maxima*)
LMG 28161^{T}	***Bombella intestini***		Belgium	Crop of a bumble bee
LMG 27436^{T} *	***"Commensalibacter intestini"***			Gut of *Drosophila melanogaster*
LMG 26838^{T}	***Endobacter medicaginis***		Zamora, Spain	Surface-sterilized nodule of *Medicago sativa*
LMG 27801^{T} *	***Gluconacetobacter aggeris***		Asuka village, Japan	Soil
LMG 27724^{T} *	***Gluconacetobacter asukensis***		Asuka village, Japan	A brown viscous gel in the stone chamber of the Kitora Tumulus
LMG 21311^{T}	***Gluconacetobacter azotocaptans***		Tapachula Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23156	*Gluconacetobacter azotocaptans*		Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23157	*Gluconacetobacter azotocaptans*		Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 1732	*Gluconacetobacter diazotrophicus*		Australia	Surface microflora of *Saccharum officinarum*
LMG 7603^{T}	***Gluconacetobacter diazotrophicus***		Alagoas, Brazil	Root of *Saccharum officinarum*
LMG 7971	*Gluconacetobacter diazotrophicus*		Pernambuco, Brazil	Stem of *Saccharum officinarum*
LMG 8065	*Gluconacetobacter diazotrophicus*		Rio de Janeiro, Brazil	Stem of *Saccharum officinarum*
LMG 8067	*Gluconacetobacter diazotrophicus*		Rio de Janeiro, Brazil	Root of *Saccharum officinarum*
LMG 22174	*Gluconacetobacter diazotrophicus*		Tamilnadu, India	Root tissue of *Oryza sativa*
LMG 25582	*Gluconacetobacter diazotrophicus*		Bois Rouge, Mauritius	Sugercane trash
LMG 25583	*Gluconacetobacter diazotrophicus*		Sodnac Quatre Bornes, Mauritius	Leaf sheath of sugercane
R-40435	*Gluconacetobacter diazotrophicus*		Mauritius	Sugarcane fields
LMG 21312^{T}	***Gluconacetobacter johannae***		Tapachula Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23153	*Gluconacetobacter johannae*		Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23154	*Gluconacetobacter johannae*		Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 1348	*Gluconacetobacter liquefaciens*		Japan	Fruit
LMG 1381^{T}	***Gluconacetobacter liquefaciens***		Japan	Dried fruit of *Diospyros* sp.
LMG 1503	*Gluconacetobacter liquefaciens*			
LMG 1509	*Gluconacetobacter liquefaciens*			Canal water
LMG 27031 *	*Gluconacetobacter liquefaciens*		Central Thailand	Flower of *Heliconia* sp.
LMG 27032 *	*Gluconacetobacter liquefaciens*		Central Thailand	*Helicona* sp.
LMG 27035 *	*Gluconacetobacter liquefaciens*		Northern Thailand	Fruit of *Manikara zapota*
LMG 19747^{T}	***Gluconacetobacter sacchari***		Queensland, Australia	Leaf sheath of sugar cane
LMG 19748	*Gluconacetobacter sacchari*		Queensland, Australia	Mealy bug
LMG 25587	*Gluconacetobacter sacchari*		Moka, Mauritius	Sugercane trash
LMG 27800^{T} *	***Gluconacetobacter takamatsuzukensis***		Japan	Soil in Takamatsuzuka Tumulus
LMG 27725^{T} *	***Gluconacetobacter tumulicola***		Asuka village, Japan	A black viscous substance in the stone chamber of the Kitora Tumulus
LMG 27802^{T} *	***Gluconacetobacter tumulisoli***		Asuka village, Japan	Clay soil in Takamatsuzuka Tumulus
LMG 1356^{T}	***Gluconobacter albidus***		Japan	*Dahlia coccinea*
LMG 1375	*Gluconobacter albidus*		Nishinomiya Japan	Fruit of *Myrica rubra*
LMG 27748^{T}	***Gluconobacter cerevisiae***		Belgium	Fermenting lambic beer
LMG 27749	*Gluconobacter cerevisiae*		Belgium	Spoiled brewer's yeast starter culture
LMG 27882	*Gluconobacter cerevisiae*		Belgium	A traditional lambic brewery
LMG 1368^{T}	***Gluconobacter cerinus***		Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1376 t1	*Gluconobacter cerinus*		Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1389	*Gluconobacter cerinus*		Herbal garden, Kyoto, Japan	Flower of *Rheum rhabarbarum*
LMG 1390	*Gluconobacter cerinus*		Herbal garden, Kyoto, Japan	Flower of *Rheum rhabarbarum*
LMG 1357 t2	*Gluconobacter frateurii*		Botanical garden, Kyoto, Japan	Flower of *Dahlia* sp.
LMG 1358	*Gluconobacter frateurii*		Botanical garden, Kyoto, Japan	Flower of *Dahlia* sp.
LMG 1365^{T}	***Gluconobacter frateurii***		Osaka, Japan	*Fragaria ananassa*
LMG 1369 t1	*Gluconobacter frateurii*		Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1371	*Gluconobacter frateurii*		Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1379	*Gluconobacter frateurii*		Osaka, Japan	Fruit of *Eriobotrya japonica*
LMG 1364	*Gluconobacter japonicus*		Osaka, Japan	*Fragaria ananassa*
LMG 1370	*Gluconobacter japonicus*		Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1373^{T}	***Gluconobacter japonicus***		Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1374	*Gluconobacter japonicus*		Nishinomiya, Japan	Fruit of *Myrica rubra*

LMG 1575	*Gluconobacter japonicus*		Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1417	*Gluconobacter japonicus* $^{\$}$	*Gluconobacter cerinus*		
LMG 26773	*Gluconobacter japonicus* $^{\#}$	*Gluconobacter nephelii*	Thailand	Rambutan (*Nephelium lappaceum*)
LMG 27103 *	*Gluconobacter japonicus* $^{\#}$	*Gluconobacter nephelii*	Thailand	Rambutan (*Nephelium lappaceum*)
NBRC 103476 *	*Gluconobacter japonicus* $^{\#}$	*Gluconobacter frateurii*	Sakaerat, Thailand	Flower
NBRC 103491 *	*Gluconobacter japonicus* $^{\#}$	*Gluconobacter frateurii*	Tong Pha Phum, Thailand	Fruit
LMG 26774^{T}	***Gluconobacter kanchanaburiensis***		Tong Pha Phum, Thailand	Fermented fruit of *Artocarpus heterophyllus*
NBRC 103588 *	*Gluconobacter kanchanaburiensis*		Tong Pha Phum, Thailand	Fermented fruit of *Artocarpus heterophyllus*
LMG 1367 t1^{T}	***Gluconobacter kondonii***		Osaka, Japan	*Fragaria ananassa*
NBRC 103510 *	*Gluconobacter kondonii* $^{\$}$	*Gluconobacter albidus*	Tong Pha Phum, Thailand	Flower of *Mucuna pruriens*
LMG 27435^{T}	**"*Gluconobacter morbifer*"**			Gut of *Drosophila melanogaster*
LMG 1359	*Gluconobacter oxydans*			
LMG 1385	*Gluconobacter oxydans*		Osaka, Japan	Dried fruit of *Diospyros* sp.
LMG 1393	*Gluconobacter oxydans*			
LMG 1395	*Gluconobacter oxydans*		Botanical garden, Kyoto, Japan	Flower of *Liatris scariosa*
LMG 1408^{T}	***Gluconobacter oxydans***			Beer
LMG 1424	*Gluconobacter oxydans*			
LMG 1674	*Gluconobacter oxydans*		Delft, The Netherlands	Beer
LMG 27012 *	*Gluconobacter oxydans* $^{\#}$	*Gluconobacter uchimurae*	Thailand	Rakam fruit (*Zalacca wallichiana*)
LMG 27013 *	*Gluconobacter oxydans* $^{\#}$	*Gluconobacter uchimurae*	Thailand	Litchi fruit
LMG 27014 *	*Gluconobacter oxydans* $^{\#}$	*Gluconobacter uchimurae*	Bangkok, Thailand	Jujube fruit
LMG 27015 *	*Gluconobacter oxydans* $^{\#}$	*Gluconobacter uchimurae*	Bangkok, Thailand	Longan fruit
LMG 27054 *	*Gluconobacter oxydans*		Thailand	
LMG 1418^{T}	***Gluconobacter roseus***		Tokyo, Japan	Fruit of *Diospyros* sp.
LMG 1414^{T}	***Gluconobacter sphaericus***		Japan	Grapes
NBRC 103509 *	*Gluconobacter sphaericus* $^{\$}$	*Gluconobacter albidus*	Tong Pha Phum, Thailand	Fruit of apple guava (*Psidium guajava*)
NBRC 103521 *	*Gluconobacter sphaericus* $^{\$}$	*Gluconobacter albidus*	Tong Pha Phum, Thailand	Fruit
LMG 1486	*Gluconobacter thailandicus*		Osaka, Japan	*Fragaria ananassa*
LMG 1487	*Gluconobacter thailandicus*		Osaka, Japan	*Fragaria ananassa*
LMG 1488	*Gluconobacter thailandicus*		Osaka, Japan	Fruit of *Prunus tomentosa*
LMG 1491	*Gluconobacter thailandicus* $^{\$}$	*Gluconobacter cerinus*	Osaka, Japan	*Diospyros* sp.
LMG 23137^{T}	***Gluconobacter thailandicus***		Bangkok, Thailand	Flower of the Indian cork tree (*Millingtonia hortensi.*
LMG 23138	*Gluconobacter thailandicus*		Bangkok, Thailand	Flower of glossy ixora (*Ixora lobbii*)
LMG 27016^{T} *	***Gluconobacter wancherniae***		Nakhon Ratchasima, Thailand	Seed
NBRC 103582 *	*Gluconobacter wancherniae*		Nakhon Ratchasima, Thailand	Fruit
LMG 24392^{T}	***Granulibacter bethesdensis***		Bethesda, United States	Patient with chronic granulomatous disease, lymph n
LMG 1380	*Komagataeibacter europaeus*		Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1510	*Komagataeibacter europaeus*		Copenhagen, Denmark	Vinegar
LMG 1521	*Komagataeibacter europaeus*		Nairobi, Kenya	Vinegar brew
LMG 18494	*Komagataeibacter europaeus*		Ljubljana Slovenia	Red wine vinegar produced in submerged bioreactor
LMG 18890^{T}	***Komagataeibacter europaeus***		Esslingen, Germany	Submerged culture vinegar generator
LMG 20956	*Komagataeibacter europaeus*		Ljubljana, Slovenia	Cider vinegar produced in industrial submerged biorc
LMG 1524	*Komagataeibacter hansenii*		Jerusalem, Israel	Vinegar
LMG 1527^{T}	***Komagataeibacter hansenii***			Celluloseless mutant 1 from NCIB 8745 from vinega
LMG 1528	*Komagataeibacter hansenii*			Celluloseless mutant 2 from NCIB 8745 from vinega
LMG 23726	*Komagataeibacter hansenii*		India	Kombucha tea ferment
LMG 27060 *	*Komagataeibacter hansenii*			
LMG 27286 *	*Komagataeibacter hansenii*		Northern Thailand	Beleric myrobalan
LMG 18909^{T}	***Komagataeibacter intermedius***		Switzerland	Commercially available kombucha
LMG 26206^{T}	***Komagataeibacter kakiaceti***		Kumamoto Prefecture, Japan	Kaki vinegar
LMG 27001 *	*Komagataeibacter kakiaceti*		Kumamoto Prefecture, Japan	Kaki vinegar
LMG 1529^{T}	***Komagataeibacter maltaceti***		United Kingdom	malt vinegar brewery acetifiers
LMG 1693^{T}	***Komagataeibacter medellinensis***		Japan	Vinegar
LMG 1536^{T}	***Komagataeibacter nataicola***		Manila, Philippines	Nata de coco
LMG 1517	*Komagataeibacter oboediens*			
LMG 1688	*Komagataeibacter oboediens*			
LMG 1689	*Komagataeibacter oboediens*			
LMG 18849^{T}	***Komagataeibacter oboediens***		Esslingen, Germany	Red wine vinegar fermentation
LMG 18907	*Komagataeibacter oboediens*		Ljubljana, Slovenia	Cider vinegar produced in submerged bioreactor
LMG 18908	*Komagataeibacter oboediens*		Ljubljana, Slovenia	Cider vinegar produced in submerged bioreactor
LMG 22126^{T}	***Komagataeibacter rhaeticus***		Val Venosta South Tyrol, Italy	Organic apple juice
LMG 27048 *	*Komagataeibacter rhaeticus*		Central Thailand	Rambutan
LMG 1582^{T}	***Komagataeibacter saccharivorans***		Germany	Beet juice
LMG 1584	*Komagataeibacter saccharivorans*			
LMG 18788^{T}	***Komagataeibacter sucrofermentans***		Tokyo, Japan	Black cherry
LMG 27287 *	*Komagataeibacter sucrofermentans* $^{\$}$	*Komagataeibacter nataicola*	Central Thailand	Fetid passinflower
LMG 25	*Komagataeibacter swingsii*			

LMG 22125^{T}	***Komagataeibacter swingsii***		Val Venosta South Tyrol, Italy	Organic apple juice
LMG 1515^{T}	***Komagataeibacter xylinus***			Mountain ash berries
LMG 1518	*Komagataeibacter xylinus*		Verona	
LMG 21812^{T}	***Kozakia baliensis***		Bali, Indonesia	Palm brown sugar
LMG 27017 *	*Kozakia baliensis*		Yogyakarta, Indonesia	Ragi (starter)
LMG 27018 *	*Kozakia baliensis*		Yogyakarta, Indonesia	Ragi (starter)
LMG 27019 *	*Kozakia baliensis*		Yogyakarta, Indonesia	Ragi (starter)
LMG 24037^{T}	***Neoasaia chiangmaiensis***		Chiang-Mai, Thailand	Flower of red ginger (*Alpinia purpurata*)
LMG 27020^{T} *	***Neokomagataea tanensis***		Thailand	Flower of candle bush (*Senna alata*)
LMG 27021^{T} *	***Neokomagataea thailandica***		Thailand	Flower of spanish flag (*Lantana camera*)
LMG 23170^{T}	***Saccharibacter floricola***		Kanagawa Prefecture, Japan	Pollen
LMG 21291^{T}	***Swaminathania salitolerans***		India	*Porteresia coarctata*
LMG 27022^{T} *	***Tanticharoenia sakaeratensis***		Thailand	Soil
NBRC 103194 *	*Tanticharoenia sakaeratensis*		Thailand	Soil
NBRC 103195 *	*Tanticharoenia sakaeratensis*		Thailand	Soil

Type strains are marked in bold.

LMG, BCCM/LMG Bacteria Collection, Ghent University, Belgium; **NBRC**, NITE Biological Resource Center, Department of Biotechnology, National Institute of Technology and Evaluation, Kisarazu, Japan; **R-**, Research Collection of LM-UGent, Ghent University, Belgium.

* Strains requested from other culture collections (BCC, NBRC, DSMZ and Riken BRC) or researchers in the frame of this study.

$ Reclassified based on AFLP and/or MLSA data obtained in this study.

Reclassified based on data obtained in this study through a polyphasic approach.

Table S3.2. List of the poorly characterized BCCM/LMG strains selected for identification through MALDI-TOF MS.

Strain number	Species name according to catalogue	Identification by MALDI-TOF MS, 16S rRNA, housekeeping gene sequence or ANI data	Geographic origin	Biological origin
1261	*Acetobacter aceti*	*Acetobacter aceti*		Beech-wood shavings of vinegar plant
1372	*Acetobacter* sp.	*Acetobacter aceti*		
1525	*Acetobacter aceti*	*Acetobacter aceti*	Zwolle, The Netherlands	Quick vinegar
1695	*Acetobacter* sp.	*Acetobacter aceti*		
18492	*Acetobacter aceti*	*Acetobacter aceti*	Kolinska, Slovenia	Cider vinegar
1544	*Acetobacter* sp.	*Acetobacter cerevisiae*		Beer
1546	*Acetobacter acetosus;*	*Acetobacter cerevisiae*		
1549	*Acetobacter pasteurianus*	*Acetobacter cerevisiae*		Top fermentation storage beer
1585	*Acetobacter* sp.	*Acetobacter cerevisiae*	Ireland	Beer
1596	*Acetobacter* sp.	*Acetobacter cerevisiae*		
1600	*Acetobacter pasteurianus*	*Acetobacter cerevisiae*	United Kingdom	Brewers' yeast and beer
1602	*Acetobacter pasteurianus*	*Acetobacter cerevisiae*	United Kingdom	Brewers' yeast and beer
1643	*Acetobacter cerevisiae*	*Acetobacter cerevisiae*		Manufacture of vinegar
1645	*Acetobacter rancens var. saccharov*	*Acetobacter cerevisiae*		
1647	*Acetobacter cerevisiae*	*Acetobacter cerevisiae*	The Netherlands	Beer
1652	*Acetobacter* sp.	*Acetobacter cerevisiae*	Ireland	Beer
1653	*Acetobacter* sp.	*Acetobacter cerevisiae*	Ireland	Beer
1654	*Acetobacter* sp.	*Acetobacter cerevisiae*	Ireland	Beer
1655	*Acetobacter* sp.	*Acetobacter cerevisiae*	United Kingdom	Farm cider
1684 t1	*Acetobacter* sp.	*Acetobacter cerevisiae*		Beer
1684 t2	*Acetobacter* sp.	*Acetobacter cerevisiae*		Beer
1685 t1	*Gluconobacter oxydans*	*Acetobacter cerevisiae*		
1705	*Acetobacter* sp.	*Acetobacter cerevisiae*	United Kingdom	Brewery
1665	*Acetobacter* sp.	*Acetobacter fabarum*		Experimental vinegar production from fermented apple juice
1607	*Acetobacter pasteurianus*	*Acetobacter indonesiensis*		Vinegar
1579^{T}	*Acetobacter lovaniensis*	*Acetobacter lovaniensis*	Becquevoort, Belgium	Sewage on soil
1502 t1	*Acetobacter pasteurianus*	*Acetobacter malorum*		
1597	*Acetobacter pasteurianus*	*Acetobacter malorum*	United Kingdom	Brewers' yeast and beer
1598	*Acetobacter pasteurianus*	*Acetobacter malorum*	United Kingdom	Brewers' yeast and beer
1642	*Acetobacter* sp.	*Acetobacter malorum*		
1646 t1	Unidentified	*Acetobacter malorum*		
1646 t2	Unidentified	*Acetobacter malorum*		
1651	*Acetobacter* sp.	*Acetobacter malorum*	United Kingdom	Sample of bottled ale
1698	*Acetobacter pasteurianus*	*Acetobacter malorum*	United Kingdom	Brewery
1700	Unidentified	*Acetobacter malorum*	United Kingdom	Brewery
1803	*Acetobacter pasteurianus*	*Acetobacter okinawensis*	United Kingdom	*Malus sylvestris*
1804	*Acetobacter pasteurianus*	*Acetobacter okinawensis*	United Kingdom	*Malus sylvestris*
1586	*Acetobacter* sp.	*Acetobacter oeni* $^{\$}$	United Kingdom	Apple juice
1576	*Acetobacter pasteurianus*	*Acetobacter orientalis*		
1664	*Acetobacter pasteurianus*	*Acetobacter orientalis*	United Kingdom	Fermenting putrified meat sample
1671	*Acetobacter pasteurianus*	*Acetobacter orientalis*	United Kingdom	Fermenting putrified meat sample
1672	*Acetobacter pasteurianus*	*Acetobacter orientalis*	United Kingdom	Fermenting putrified meat sample
1282	*Acetobacter lovaniensis*	*Acetobacter orleanensis*	Belgium	Beer
1497 t1	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*		
1497 t2	*Acetobacter rancens*	*Acetobacter pasteurianus*		
1513	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	Aichi, Japan	Film in fermentor of rice vinegar
1514	*Acetobacter lovaniensis*	*Acetobacter pasteurianus*	Aichi, Japan	Film in fermentor of rice vinegar
1540	*Acetobacter* sp.	*Acetobacter pasteurianus*	Japan	
1541	*Acetobacter* sp.	*Acetobacter pasteurianus*	Japan	
1543	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	East Africa	Vinegar brews
1548	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1550	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1551	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*		
1554	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*		
1556	*Acetobacter* sp.	*Acetobacter pasteurianus* $^{\$}$		
1577	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1594	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*		

1603	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*		Vinegar brews
1605	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	East Africa	Vinegar brews
1606	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	The Netherlands	Beer
1609	*Gluconobacter oxydans*	*Acetobacter pasteurianus*		
1610	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*		
1611	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1612	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1613	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	The Netherlands	Beer
1615 t1	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	The Netherlands	Beer
1616	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	The Netherlands	Beer
1620	*Acetobacter* sp.	*Acetobacter pasteurianus* [$]		Malt vinegar brewery acetifiers
1622	*Acetobacter* sp.	*Acetobacter pasteurianus*		Malt vinegar brewery acetifiers
1623	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1624 t1	Unidentified	*Acetobacter pasteurianus*		
1631	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	Recife, Brazil	Acidified *Saccharum officinarum* juice
1632	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	Africa	African vinegar
1638	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1639	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	The Netherlands	Beer
1640	*Acetobacter* sp.	*Acetobacter pasteurianus*		
1649 t1	*Acetobacter rancens*	*Acetobacter pasteurianus*		
1649 t2	*Acetobacter rancens*	*Acetobacter pasteurianus*		
1650	*Gluconobacter oxydans*	*Acetobacter pasteurianus*		
1666	*Acetobacter* sp.	*Acetobacter pasteurianus*		Fermented alcoholic mash
1685 t2	Unidentified	*Acetobacter pasteurianus* [¶]		
26310	*Acetobacter pasteurianus*	*Acetobacter pasteurianus*	Switzerland	Red wine vinegar (submerged methodology)
6039	Unidentified	*Acetobacter pasteurianus*		
1501	*Acetobacter* sp.	*Acetobacter sicerae*		
1508	*Acetobacter aceti*	*Acetobacter sicerae*		
1530	*Acetobacter* sp.	*Acetobacter sicerae*		
1670	*Acetobacter aceti*	*Acetobacter sicerae*	United Kingdom	Fermenting putrified meat sample
5	*Acetobacter aceti*	*Acetobacter sicerae*	Belgium	Beer
1627	*Acetobacter pasteurianus*	*Acetobacter sp.* nov. I [$]		
1636	*Acetobacter* sp.	*Acetobacter* sp. nov. II [$]		Starch producing mutant of an *Acetobacter*
1637	Unidentified	*Acetobacter* sp. nov. II [¶]		
1506	*Acetobacter aceti*	*Acetobacter* sp. nov. III [$]	Belgium	Beer
1706	*Acetobacter* sp.	*Acetobacter* sp. nov. III [¶]	United Kingdom	Brewery
1426	*Gluconobacter* sp.	*Asaia astilbes*	Aalter, Belgium	*Solidago canadensis*
1760	Unidentified	*Asaia platycodi*	The Netherlands	Apple soft drink
1762	Unidentified	*Asaia platycodi*	The Netherlands	Orange stilldrink
1453	*Gluconobacter oxydans*	*Asaia* sp. nov. [$]		
1352	*Acetobacter* sp.	*Bombella* sp. nov. [¶]	Madrid area, Spain	Beehive
1354	*Acetobacter* sp.	*Bombella* sp. nov. [+]	Madrid area, Spain	Beehive
1736 t1	*Gluconacetobacter sacchari*	*Gluconacetobacter diazotrophicus*	Australia	*Saccharum officinarum*, surface microflora
1733	*Gluconacetobacter diazotrophicus*	*Gluconacetobacter diazotrophicus*	Australia	*Saccharum officinarum*, surface microflora
1734	*Gluconacetobacter diazotrophicus*	*Gluconacetobacter diazotrophicus*	Australia	*Saccharum officinarum*, surface microflora
7602	*Gluconacetobacter diazotrophicus*	*Gluconacetobacter diazotrophicus*	Alagoas, Brazil	*Saccharum officinarum*, root
8066	*Gluconacetobacter diazotrophicus*	*Gluconacetobacter diazotrophicus*	Alagoas, Brazil	*Saccharum officinarum*, root
8068	*Gluconacetobacter diazotrophicus*	*Gluconacetobacter diazotrophicus*	Rio de Janeiro, Brazil	*Saccharum officinarum*, rhizosphere
1347	*Gluconacetobacter liquefaciens*	*Gluconacetobacter liquefaciens*	Japan	Fruit
1388	*Gluconacetobacter liquefaciens*	*Gluconacetobacter liquefaciens*	Japan	Fruit
1532	*Gluconacetobacter liquefaciens*	*Gluconacetobacter liquefaciens*	Japan	Fruit
26312	*Gluconacetobacter oboediens*	*Gluconacetobacter oboediens*	Switzerland	Spirit vinegar (submerged methodology)
1736 t2	*Gluconacetobacter liquefaciens*	*Gluconacetobacter sacchari*	Australia	*Saccharum officinarum*, surface microflora
1728	*Gluconacetobacter liquefaciens*	*Gluconacetobacter takamatsuzukensis*	Hawaii, United States	*Ananas comosus*, pink diseased fruit
1409	*Gluconobacter oxydans*	*Gluconobacter albidus*		Cider
1431 t1	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1431 t2	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1432	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1434	*Gluconobacter oxydans*	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1440	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)

1442	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1443	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1450	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1455	*Gluconobacter* sp.	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1559	*Gluconobacter oxydans*	*Gluconobacter albidus*	Japan	*Lilium auratum*
1768	*Gluconobacter oxydans*	*Gluconobacter albidus* *	The Netherlands	Orange lemonade
1350	*Gluconobacter oxydans*	*Gluconobacter cerevisiae*		
1351	*Gluconobacter oxydans*	*Gluconobacter cerevisiae*		
1430	*Gluconobacter* sp.	*Gluconobacter cerevisiae*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1704	*Gluconobacter* sp.	*Gluconobacter cerevisiae*	United Kingdom	Brewery
1707	*Gluconobacter* sp.	*Gluconobacter cerevisiae*	United Kingdom	Brewery
1362	*Gluconobacter cerinus*	*Gluconobacter cerinus*	Japan	*Malus* sp.
1376 t2	*Gluconobacter cerinus*	*Gluconobacter cerinus*	Nishinomiya, Japan	*Myrica rubra*, fruit
1416	*Gluconobacter cerinus*	*Gluconobacter cerinus*		
1420	*Gluconobacter cerinus*	*Gluconobacter cerinus*		
1425	*Gluconobacter oxydans*	*Gluconobacter cerinus*		
1427	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1428	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1429	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1433	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1435	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1436	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1437	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1438	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1439	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1441	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1444	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1445	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1446	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1447 t1	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1447 t2	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1448	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1449	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1451	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1452	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	*Helenium* sp.
1454	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1456	*Gluconobacter* sp.	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
1457	*Gluconobacter* sp.	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
1458	*Gluconobacter* sp.	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
1459	*Gluconobacter* sp.	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
1460	*Gluconobacter* sp.	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
1461	*Gluconobacter* sp.	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
1462	*Gluconobacter* sp.	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
1463	*Gluconobacter* sp.	*Gluconobacter cerinus*	near Zottegem, Belgium	Honeybee (*Apis mellifera*)
1464	*Gluconobacter* sp.	*Gluconobacter cerinus*	Zottegem, Belgium	Honeybee (*Apis mellifera*)
1465	*Gluconobacter* sp.	*Gluconobacter cerinus*	Zottegem, Belgium	Honeybee (*Apis mellifera*)
1475	*Gluconobacter cerinus*	*Gluconobacter cerinus*	Zottegem, Belgium	Honeybee (*Apis mellifera*)
1679 t1	*Gluconobacter cerinus*	*Gluconobacter cerinus*	Leiden, The Netherlands	Beer
1679 t2	*Gluconobacter cerinus*	*Gluconobacter cerinus*	Leiden, The Netherlands	Beer
1718	*Gluconobacter* sp.	*Gluconobacter cerinus*	Alberton, South Africa	Lager beer at fermentation stage
1747	*Gluconobacter* sp.	*Gluconobacter cerinus*	Belgium	*Malus* sp.
1749	*Gluconobacter cerinus*	*Gluconobacter cerinus*	Ghent, Belgium	Rotting apple
1763	*Gluconobacter cerinus*	*Gluconobacter cerinus*	The Netherlands	Orange lemonade
1766	*Gluconobacter cerinus*	*Gluconobacter cerinus*	The Netherlands	Raspberry stilldrink
1767	*Gluconobacter cerinus*	*Gluconobacter cerinus*	The Netherlands	Black current lemonade
1737	*Gluconobacter cerinus*	*Gluconobacter frateurii*	Ghent, Belgium	Rotting pear
1363	*Gluconobacter cerinus*	*Gluconobacter frateurii*	Osaka, Japan	*Fragaria ananassa*
1366	*Gluconobacter frateurii*	*Gluconobacter frateurii*	Osaka, Japan	*Fragaria ananassa*
1369 t2	*Gluconobacter frateurii*	*Gluconobacter frateurii*	Osaka, Japan	*Prunus* sp. (cherry)
1378	*Gluconobacter cerinus*	*Gluconobacter frateurii*	Osaka, Japan	*Eriobotrya japonica*, fruit
1419	*Gluconobacter frateurii*	*Gluconobacter frateurii*		

1421	*Gluconobacter frateurii*	*Gluconobacter frateurii*		
1492	*Gluconobacter cerinus*	*Gluconobacter frateurii*	Osaka, Japan	*Diospyros* sp.
1570	*Gluconobacter cerinus*	*Gluconobacter frateurii*	Osaka, Japan	*Fragaria ananassa*
1656	*Gluconobacter frateurii*	*Gluconobacter frateurii*		
1738	*Gluconobacter* sp.	*Gluconobacter frateurii*	Ghent, Belgium	*Pyrus communis*
1281	*Gluconobacter japonicus*	*Gluconobacter japonicus*	Nishinomiya, Japan	*Myrica rubra*, fruit
1383	*Gluconacetobacter liquefaciens*	*Gluconobacter japonicus*	Japan	Fruit
1397	*Gluconobacter cerinus*	*Gluconobacter japonicus*		
1407	*Gluconobacter cerinus*	*Gluconobacter japonicus*	Leuven, Belgium	Bakers' yeast
1574	*Gluconobacter cerinus*	*Gluconobacter japonicus*		
1675	*Gluconobacter cerinus*	*Gluconobacter japonicus*	Leuven, Belgium	Bakers' yeast
1678 t1	*Gluconobacter cerinus*	*Gluconobacter japonicus*	Delft, The Netherlands	Beer
1678 t2	*Gluconobacter cerinus*	*Gluconobacter japonicus*	Delft, The Netherlands	Beer
1355	*Gluconobacter albidus*	*Gluconobacter oxydans*	Japan	*Dahlia coccinea*
1384	*Gluconobacter* sp.	*Gluconobacter oxydans*		
1394	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1398	*Gluconobacter oxydans*	*Gluconobacter oxydans*	United Kingdom	Beer
1400	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1401	*Gluconobacter oxydans*	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
1402	*Gluconobacter oxydans*	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
1403	*Gluconobacter oxydans*	*Gluconobacter oxydans*		Derived from ATCC 621
1404	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1405	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1410	*Gluconobacter* sp.	*Gluconobacter oxydans*		
1411	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1413	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1422	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1423	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1484	*Gluconobacter oxydans*	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
1485	*Gluconobacter* sp.	*Gluconobacter oxydans*		
1494	*Gluconobacter oxydans*	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
1516	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1519	*Gluconobacter oxydans*	*Gluconobacter oxydans*	United Kingdom	Ropy sample of top fermented beer
1533	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1569	*Gluconobacter oxydans*	*Gluconobacter oxydans*	United Kingdom	Beer
1581	*Gluconobacter oxydans*	*Gluconobacter oxydans*	Delft, The Netherlands	Beer
1589[T]	*Gluconobacter oxydans*	*Gluconobacter oxydans*		Beer
1673	*Gluconobacter oxydans*	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
1677	*Gluconobacter oxydans*	*Gluconobacter oxydans*	Delft, The Netherlands	Beer
1681	*Gluconobacter oxydans*	*Gluconobacter oxydans*		derived from ATCC 621
1683	*Gluconobacter oxydans*	*Gluconobacter oxydans*		Irish beer
1691	*Gluconobacter oxydans*	*Gluconobacter oxydans*		
1765	*Gluconobacter oxydans*	*Gluconobacter oxydans*	The Netherlands	Lemon lemonade
1785	*Gluconobacter* sp.	*Gluconobacter oxydans*	Ireland	Beer
1680	*Gluconobacter oxydans*	*Gluconobacter oxydans*	South Africa	Sour porridge
1386	*Gluconobacter* sp.	*Gluconobacter oxydans* *	Osaka, Japan	*Diospyros* sp., dried fruit
1399	*Gluconobacter oxydans*	*Gluconobacter oxydans* *		Beer
1406	*Gluconobacter oxydans*	*Gluconobacter oxydans* *		Ropy beer
1676	*Gluconobacter oxydans*	*Gluconobacter oxydans* *	Leuven, Belgium	Beer
1764	*Gluconobacter oxydans*	*Gluconobacter oxydans* [#]	The Netherlands	Cider lemonade
1412	*Gluconobacter roseus*	*Gluconobacter roseus*	Tokyo, Japan	*Diospyros* sp., fruit
1744	*Gluconobacter oxydans*	*Gluconobacter sphaericus*	Ghent, Belgium	Rotting pear
1377	*Gluconobacter cerinus*	*Gluconobacter thailandicus*		
1489	*Gluconobacter cerinus*	*Gluconobacter thailandicus*	Osaka, Japan	*Prunus tomentosa*, fruit
1490	*Gluconobacter cerinus*	*Gluconobacter thailandicus*	Osaka, Japan	*Prunus tomentosa*, fruit
1493	*Gluconobacter cerinus*	*Gluconobacter thailandicus*	Osaka, Japan	*Diospyros* sp.
1739	*Gluconobacter* sp.	*Gluconobacter thailandicus*	Ghent, Belgium	*Pyrus communis*
1740	*Gluconobacter* sp.	*Gluconobacter thailandicus*	Ghent, Belgium	*Pyrus communis*
1742	*Gluconobacter* sp.	*Gluconobacter thailandicus*	Ghent, Belgium	*Pyrus communis*
1743	*Gluconobacter cerinus*	*Gluconobacter thailandicus* *	Ghent, Belgium	Rotting pear

1741	*Gluconobacter cerinus*	*Gluconobacter thailandicus* *	Ghent, Belgium	Rotting pear
1745	*Gluconobacter oxydans*	*Gluconobacter* sp. +	Ghent, Belgium	Rotting apple
12	*Acetobacter sp.*	*Komagataeibacter europaeus*		
1520	*Acetobacter* sp.	*Komagataeibacter europaeus*	Kenya	Vinegar brews
1523	*Gluconacetobacter xylinus*	*Komagataeibacter europaeus*	East Africa	Vinegar
1641	*Gluconobacter* sp.	*Komagataeibacter europaeus*		African vinegar brew
1690	*Acetobacter* sp.	*Komagataeibacter europaeus*		
1696	*Gluconacetobacter europaeus*	*Komagataeibacter europaeus*	Nishinomiya, Japan	*Myrica rubra*, fruit
26311	*Gluconacetobacter europaeus*	*Komagataeibacter europaeus*	Switzerland	Red wine vinegar (submerged methodology)
1526	*Acetobacter* sp.	*Komagataeibacter medellinensis*		Beer
1692	*Acetobacter aceti*	*Komagataeibacter medellinensis*		
1522	*Acetobacter aceti subsp. xylinus;*	*Komagataeibacter oboediens*		
27414	*Gluconacetobacter rhaeticus*	*Komagataeibacter rhaeticus*		
27415	*Gluconacetobacter rhaeticus*	*Komagataeibacter rhaeticus*		
1502 t2	*Acetobacter* sp.	*Komagataeibacter saccharivorans*		
1538	*Gluconacetobacter xylinus*	*Komagataeibacter xylinus*		
1353	*Gluconobacter* sp. SU	*Pantoea* sp. $	Near Madrid, Spain	Beehive
1757	Unidentified	*Rosenbergiella collisarenosi* +		
1752	Unidentified	*Enterococcus* sp. +		
1573	*Acetobacter fragum*	*Tatumella* sp.		
1723	Unidentified	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
1725	Unidentified	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
1729	Unidentified	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
1770	Unidentified	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
1771	Unidentified	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
1773	Unidentified	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit

Strain number followed by "t" refers to different colony types with different MALDI-TOF MS profiles.

[+] Identified by partial 16S rRNA gene sequence analysis.

[$] Identified by housekeeping gene sequence analysis.

[*] Identified by ANI data.

[#] Identified by ANI data, in combination with MLSA and phenotypic data.

[¶] Identified by MALDI after identification of strains of the same MALDI cluster through housekeeping gene sequence analysis.

* Identified by MALDI after identification of strains of the same MALDI cluster through ANI data.

Table S3.3. Results of reciprocal DNA-DNA hybridizations with (A) the cocoa bean isolates R-49105, R-49109, R-49119 with *Acetobacter persici* LMG 26458^T and *Acetobacter okinawensis* LMG 26457^T (B) *Gluconobacter japonicus* strains LMG 1373^T and LMG 1417, *Gluconobacter nephelii* strains LMG 26773^T and R-49733, and the cocoa bean isolate R-49117.

(A.)

Species	Strain	**LMG 26458^T**	R-49105	R-49109
A. persici	**LMG 26458^T**	100		
A. persici	R-49105	76 (±13.5)	100	
A. persici	R-49109	78 (±13.0)	81 (±3.0)	100

Species	Strain	**LMG 26457^T**	R-49119
A. okinawensis	**LMG 26457^T**	100	
A. okinawensis	R-49119	89 (±12.0)	100

(B.)

Species	Strain	**LMG 1373^T**	LMG 1417	R-49117	**LMG 26773^T**	R-49733
G. japonicus	**LMG 1373^T**	100				
G. japonicus	LMG 1417	65 (±7.5)	100			
G. japonicus/G. nephelii	R-49117	58 (±7.5)	67 (±1.5)	100		
G. nephelii	**LMG 26773^T**	64 (±7.5)	68 (±7.0)	67 (±12.5)	100	
G.nephelii	R-49733	66 (±4.0)	69 (±8.5)	63 (±3.5)	73 (±6.5)	100

Table S3.4. Accession numbers of housekeeping genes sequences of *dnaK*, *groEL* and *rpoB* obtained in this study.

Taxon	LMG no.	Gene				
		dnaK	*groEL*	*rpoB*	16S rRNA	tartronate semialdehyde reductase*
Asaia sp. nov.	LMG 1453	KR677140	KR677134			
Acetobacter sp. nov. I	LMG 1627	KR677146	KR677137	KR677149		
Acetobacter sp. nov. II	LMG 1636	KR677147	KR677138	KR677150		
Acetobacter sp. nov. III	LMG 1506	KR677141	KR677135			
Acetobacter pasteurianus	LMG 1556	KR677143				
Acetobacter pasteurianus	LMG 1620	KR677145				
Acetobacter oeni	LMG 1586	KR677144		KR677148		
Gluconobacter sp. nov.	LMG 1745				KT366868	
Bombella sp. nov.	LMG 1354				KT366867	
Enterococcus sp.	LMG 1752				KT366869	
Rosenbergiella sp.	LMG 1757				KR677139	
Pantoea sp.	LMG 1353					KT387274

*Sequence obtained by amplification using primer combination of dnaK-01-F (5'-CTGCGCATCATCAACGAGCC-3') and dnaK-02-R (5'-CTCACGCTCGCCCTGATAGA-3').

Table S3.5. General features of genomes sequenced in this study.

Taxon	LMG number	NCBI accession no.	Contigs	Size (bp)	G+C (%)
A. cerevisiae	LMG 1625^T	LHZA00000000	157	3,088,168	58.0
A. cerevisiae	LMG 1545	LIAA00000000	108	3,323,579	57.7
A. cerevisiae	LMG 1608	LHZY00000000	177	3,225,161	57.7
A. cerevisiae	LMG 1699	LHZX00000000	319	3,641,855	57.3
A. pasteurianus	LMG 1604	LHZZ00000000	654	3,281,630	58.0
A. pasteurianus	LMG 1552	LHZF00000000	176	3,462,961	57.8
A. malorum	LMG 1746^T	LHZC00000000	57	3,833,577	56.7
A. orleanensis	LMG 1583^T	LHZV00000000	67	3,007,922	56.4
A. tropicalis	LMG 19825^T	LHZQ00000000	222	3,557,538	55.8
A. tropicalis	LMG 1663	LHZT00000000	132	3,769,713	55.6
A. senegalensis	LMG 23690^T	LHZU00000000	148	3,928,855	55.6
G. oxydans	LMG 1406	LHZL00000000	171	2,754,942	60.9
G. oxydans	LMG 1399	LHZE00000000	86	2,783,426	61.1
G. oxydans	LMG 1676	LHZG00000000	180	2,819,278	61.0
G. oxydans	LMG 1386	LHZD00000000	22	2,857,977	61.0
G. oxydans	LMG 1764	LHZB00000000	135	3,642,557	60.5
G. uchimurae	LMG 27012^T	LHZW00000000	56	2,996,164	61.0
G. roseus	LMG 1418^T	LHZP00000000	29	2,881,230	59.8
G. albidus	LMG 1356^T	LHZM00000000	146	3,270,229	59.6
G. albidus	LMG 1768	LHZR00000000	114	3,661,273	59.7
G. thailandicus	LMG 23137^T	LHZS00000000	107	3,431,389	56.2
G. thailandicus	LMG 1741	LHZM00000000	90	3,677,730	56.3
G. japonicus	LMG 1373^T	LHZK00000000	128	3,139,866	56.1
G. japonicus/nephelii	R-49117	LHZH00000000	135	3,163,248	55.8
G. japonicus	LMG 1417	LHZJ00000000	85	3,040,306	55.9
G. nephelii	R-49733	LHZI00000000	1,755	3,892,362	55.7
G. nephelii	LMG 26773^T	LHZO00000000	64	3,162,930	55.8

Table S3.6. Phenotypic features of strains of *G. japonicus* and *G. nephelii*, reported useful to differentiate them at the species level according to Kommanee *et al* (2011). 1-5, *G. japonicus* LMG 1373^T, LMG 1364, LMG 1370, LMG 1374, LMG 1575; 6. cocoa bean fermentation isolate R-49117; 7-10, *G. nephelii* LMG 26773^T, LMG 27103, R-49733, R-49734. Unless indicated otherwise, data were generated in this study. +, positive; -, negative; w, weakly positive; nd, not determined.

	1	2	3	4	5	6	7	8	9	10
Acid production from										
Maltose [$]	-	-	-	w	w	nd	w	w	nd	nd
Melibiose	+	+	-[a]	w	-[a]	-	+*	w*	w	w
L-sorbose	-[a]	+	+	-[a]	-[a]	w	w*	+*	-	-
Growth on										
Maltose	w[a]	+[a]	+[a]	+[a]	w[a]	+	+	w	+	+

[$] Data for 1-5 were taken from Malimas *et al.* (2009); data for 7-8 were taken from Kommanee *et al.* (2011).

[a] Data obtained in this study differed from data reported by Malimas *et al.* (2009).

*Data obtained in this study differed from data reported by Kommanee *et al.* (2011).

Table S3.7. Phenotypic features of strains of *G. uchimurae* and *G. oxydans*, reported useful to differentiate them at the species level according to Tanasupawat *et al* (2011). 1-4, *G. uchimurae* LMG 27012^{T}, LMG 27013, LMG 27014, and LMG 27015; 5-9, *G. oxydans* LMG 1408^{T}, LMG 1406, LMG 1399, LMG 1764 and LMG 1422. Data for 1-4 were taken from Tanasupawat *et al* (2011). Data for 5-9 were obtained by Gossel in the frame of his PhD thesis Gossel (1982), unless indicated otherwise.

	1-4	5	6	7	8	9
Water-solube brown pigment	+	-	-	-	+	+
2,5-diketo-D-gluconate formation	+	-	-	-	+	+
Acid production from						
Maltose $^{+}$	-	+	-	-	-	-

$^{+}$ Data obtained in this study.

4

Acetobacter sicerae sp. nov., isolated form cider and kefir and identification of *Acetobacter* species by *dnaK*, *groEL* and *rpoB* sequence analysis

Leilei Li, Anneleen Wieme, Freek Spitaels, Tom Balzarini, Olga C. Nunes, Célia M. Manaia, Anita Van Landschoot, Luc De Vuyst, Ilse Cleenwerck, and Peter Vandamme

Redrafted from: International Journal of Systematics and Evolutionary Microbiology, 2014, 64(7), pp 2407-2415, doi: 10.1099/ijs.0.058354-0

Summary

Five acetic acid bacteria isolates, *i.e.* awK9_3, awK9_4 (= LMG 27543), awK9_5 (= LMG 28092), awK9_6 and awK9_9, obtained during a study of microorganisms present in traditionally produced kefir, were grouped on the basis of their MALDI-TOF MS profile with LMG 1530 and LMG 1531^T, two strains currently classified as *Acetobacter* sp. Phylogenetic analysis based on nearly complete 16S rRNA gene sequences as well as on concatenated partial sequences of the housekeeping genes *dnaK*, *groEL* and *rpoB*, suggested that these isolates constituted a single novel species together with LMG 1530 and LMG 1531^T in the genus *Acetobacter*, with *Acetobacter aceti*, *Acetobacter nitrogenifigens*, *Acetobacter oeni* and *Acetobacter estunensis* as nearest neighbours. Pairwise similarity of 16S rRNA gene sequences between LMG 1531^T and the type strains of the above mentioned species were 99.7 %, 99.1 %, 98.4 % and 98.2 %, respectively. DNA-DNA hybridizations confirmed that status, while AFLP™ and RAPD data suggested that LMG 1531^T, LMG 1530, LMG 27543 and LMG 28092 are at least two different strains of the new species. The major fatty acid of LMG 1531^T and LMG 27543 was $C_{18:1}$ $\omega 7c$. The major ubiquinone present was Q-9 and the DNA G+C content of LMG 1531^T and LMG 27543 were 58.3 % and 56.7 % mol, respectively. The strains were able to grow on D-fructose and D-sorbitol as single carbon source. They were also able to grow on yeast extract with 30 % D-glucose and on standard medium with a pH of 3.6 or containing 1 % NaCl. They had a weak ability to produce acid from D-arabinose.

These features enabled their differentiation from their nearest phylogenetic neighbors. The name *Acetobacter sicerae* sp. nov. is proposed with LMG 1531^T (= NCIMB 8941^T) as the type strain.

4.1. Introduction

Acetic acid bacteria (AAB) are Gram-negative, coccoid or rod-shaped, obligately aerobic bacteria that are ubiquitous in the environment. They occur in sugary and alcoholic, slightly acidic niches including several traditional fermented foods and beverages (Kersters *et al.*, 2006; Lisdiyanti *et al.*, 2003). From the latter sources, especially strains of the genus *Acetobacter* are isolated (Lisdiyanti *et al.*, 2003). Strain LMG 1531^T, a non-cellulose-producing mutant of strain LMG 1530, which was isolated from cider (Shimwell and Carr, 1958), is phenotypically similar and phylogenetically related to *Acetobacter aceti* (Cleenwerck *et al.*, 2002; Gosselé *et al.*, 1983b; Shimwell and Carr, 1958), but was excluded from that species based on AFLP and $(GTG)_5$-PCR fingerprint data, and its low DNA-DNA relatedness value (< 60 %) towards true *A. aceti* strains (Cleenwerck *et al.*, 2009; De Vuyst *et al.*, 2008; Papalexandratou *et al.*, 2009).

4.2. Methods, results and discussion

During a study of microorganisms present in a concentrated, industrially produced kefir made with syrup as additional carbon source and ready for bottling and consumption, acetic acid bacteria were isolated as follows. The kefir sample was serially diluted to 10^{-6} in physiological water (0.85 %, w/v, NaCl) and plated onto Acetic Acid Medium (AAM) agar [1 %, w/v, D-glucose; 1.5 %, w/v, bacteriological peptone (Oxoid, Basingstoke, UK); 0.8 %, w/v, yeast extract (Oxoid); 0.3 %, v/v, acetic acid; 0.5 %, v/v, ethanol; 0.32 %, v/v, hydrochloric acid and 1.5 %, w/v, agar (Lisdiyanti *et al.*, 2001)], containing 200 ppm cycloheximide and 5 ppm amphotericin B. Acetic acid, ethanol, hydrochloric acid, cycloheximide and amphotericin B were added to the isolation medium after sterilization. Inoculated media were incubated aerobically at 30 ℃ for 5 days. Isolates were dereplicated by matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS), a fast and accurate technique for identification of many bacteria

species including AAB (Andrés-Barrao *et al.*, 2013; Anhalt and Fenselau, 1975; Claydon *et al.*, 1996; Krishnamurthy and Ross, 1996), using the method described previously (Wieme *et al.*, 2012). Five isolates showed identical mass spectra with a high level of similarity towards those of LMG 1531^T and LMG 1530, which suggested relatedness at the species level (Figure 4.1 and Figure S4.1). Two of these isolates, awK9_4 and awK9_5, were selected as representative for further investigation, and deposited in the BCCM/LMG Bacteria Collection as LMG 27543 and LMG 28092, respectively.

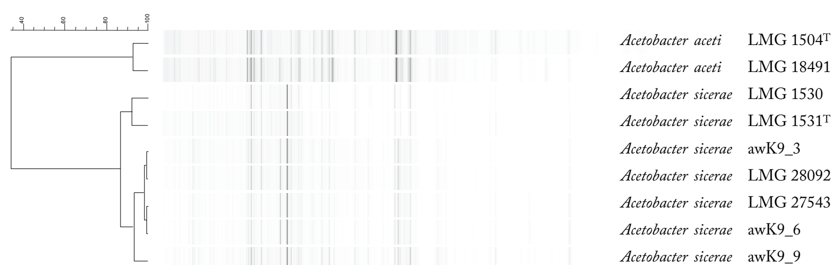


Figure 4.1. MALDI-TOF MS profiles of *Acetobacter sicerae* sp. nov. and their closest phylogenetic relative, *Acetobacter aceti*. The dendrogram was derived from unweighted pairgroup cluster analysis (UPGMA) of the fingerprints with levels of linkage expressed as Pearson correlation coefficients.

A nearly complete 16S rRNA gene sequence was determined for strains LMG 27543, LMG 28092 and LMG 1530 as described previously (Snauwaert *et al.*, 2013). The sequences were compared with 16S rRNA gene sequences of LMG 1531^T (AJ419840) and the type strains of the validly named *Acetobacter* species retrieved from the EMBL database or determined in the frame of the present study (*i.e. Acetobacter nitrogenifigens* LMG 23498^T, HG424425) using the BioNumerics 5.1 software (Applied Maths, Sint-Martens-Latem, Belgium). Strains LMG 1531^T and LMG 1530 were identified as closest relative of LMG 27543 and LMG 28092 both with >99.9 % pairwise sequence similarity, while *A. aceti* and *A. nitrogenifigens* were found as the closest established

species exhibiting 99.7 and 99.1 % pairwise sequence similarity with LMG 27543, respectively. Similarities towards other *Acetobacter* species were below 98.7 %. The 16S rRNA gene sequences of LMG 27543, LMG 28092, LMG 1530 and LMG 1531^{T}, and of all *Acetobacter* type strains were aligned against the SILVA bacteria database using the Mothur pipeline (Quast *et al.*, 2013; Schloss *et al.*, 2009). Subsequently, phylogenetic trees based on 1312-1320 nucleotides were constructed with MEGA 6 using the maximum likelihood (ML) and neighbour-joining (Felsenstein, 1981; Saitou and Nei, 1987) methods. The statistical reliability of the topology of the trees was evaluated by bootstrap analysis (Felsenstein, 1985). Both trees showed generally the same topology, and therefore only the ML tree is shown (Figure 4.2).

For species of the genus *Gluconacetobacter* and related taxa, sequences of the housekeeping genes *dnaK*, *groEL* and *rpoB* show a higher resolution than the 16S rRNA gene (Cleenwerck *et al.*, 2010). In the present study, partial sequences of these housekeeping genes were therefore determined for representative strains of all the established *Acetobacter* species and for strains LMG 27543 and LMG 1531^{T} (Table S4.1), using the approach described previously (Cleenwerck *et al.*, 2010). The obtained sequences were translated into amino acid sequences in MEGA 6 and were aligned using MUSCLE under default parameters (Edgar, 2004), after which their respective nucleotide sequences were aligned accordingly. The sequences of the three genes were concatenated (1614 bp) and a phylogenetic tree was constructed with MEGA 6 using the Maximum Likelihood model (Figure 4.3). The DNA substitution GTR+G+I was selected under the Bayesian Information Criterion (Nei and Kumar, 2000; Tamura *et al.*, 2013). A concatenated tree based on amino acid sequences (538 aa) of the above mentioned sequences was also constructed, with substitution model LG+G (Figure S4.1). Bootstrap values lower than 70 % were removed (Tindall *et al.*, 2010). *Acetobacter cibinongensis*, *Acetobacter orientalis*, *Acetobacter papayae* and *Acetobacter peroxydans* were not included in this tree as sequences of *dnaK* of the latter three species and *rpoB* of the former two species could not be obtained. A phylogenetic tree based on *groEL* sequences (528 bp) and corresponding amino acid sequences (176 aa), which includes all established *Acetobacter*

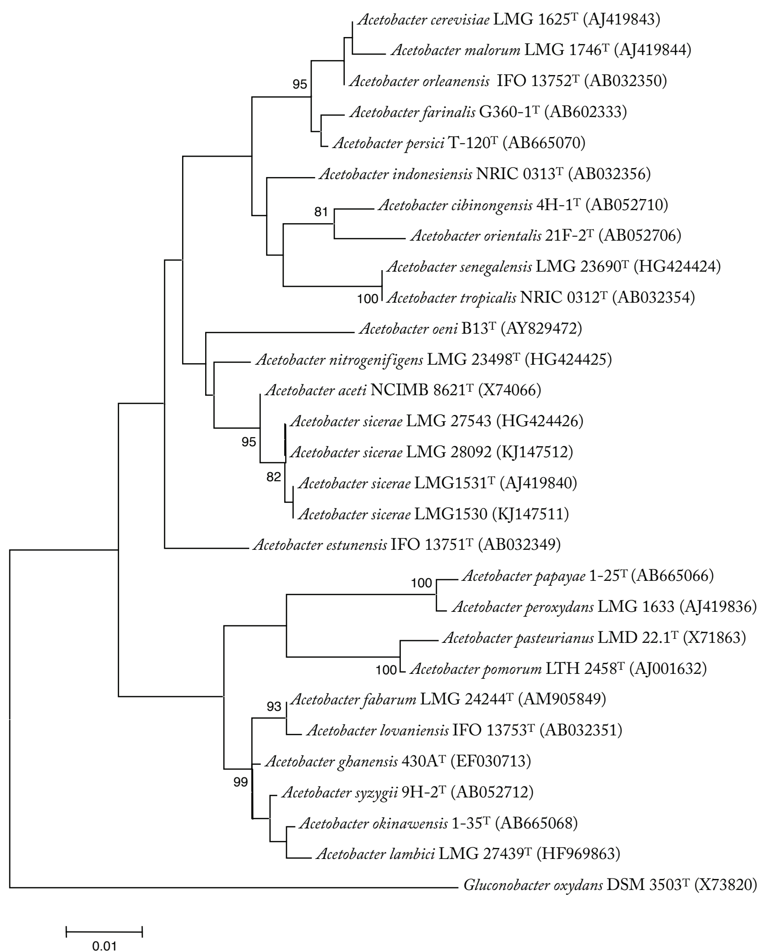


Figure 4.2. Maximum likelihood tree based on nearly complete 16S rRNA gene sequences (1312-1320 nt) showing the phylogenetic position of *Acetobacter sicerae* sp. nov. within the genus *Acetobacter*. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 1 % sequence divergence.

species, is shown in Figure S4.2, S4.3. Both nucleotide sequence based trees showed topologies similar to the 16S rRNA gene-based tree, but with a higher discriminatory power. The housekeeping gene sequences enabled differentiation of nearly all *Acetobacter* species. Only strains of the closely related species *Acetobacter cerevisiae* and *Acetobacter malorum* as well as *Acetobacter tropicalis* and *Acetobacter senegalensis* were intermixed. The concatenated tree based on amino acid sequences showed a topology similar to those of the nucleotide sequence based trees, with only a few differences, *i.e.* *Acetobacter senegalensis* and *Acetobacter tropicalis* were differentiated, while *Acetobacter lovaniensis* as well as *Acetobacter pomorum* were not differentiated from *Acetobacter fabarum* and *Acetobacter pasteurianus*, respectively. Trees based on amino acid sequences of *dnaK*, *groEL* and *rpoB* separately showed a lower taxonomical resolution (shown for *groEL* in Supplementary Figure S4.2, S4.3). These trees are less informative and thus less useful for the differentiation of AAB. In the concatenated amino acid sequences based tree, strains LMG 27543 and LMG 1531^{T} were clearly differentiated from *A. aceti*. Differentiation was noticed at amino acid positions 25 and 131 of *dnaK* and position 23 of *rpoB*. Overall, strains LMG 27543 and LMG 1531^{T} grouped together on a branch separate from all established species but close to *A. aceti*, suggesting they represented a single novel species within the genus *Acetobacter*. Additionally, their nucleotide sequences were not identical, indicating they were different strains.

Amplified fragment length polymorphism (AFLP™) DNA fingerprinting was performed on strains LMG 27543, LMG 1530 and LMG 28092 as previously described (Castro *et al.*, 2013). The obtained DNA fingerprints were compared with AFLP profiles of AAB present in a BCCM/LMG in house database (Cleenwerck *et al.*, 2009). The strains formed a cluster with LMG 1531^{T} separate from the related species (Figure 4.4), confirming the MLSA results. In addition, the cluster showed two distinct DNA fingerprint types (with LMG 1530 and LMG 1531^{T} forming the first type, and LMG 27543 and LMG 28092 forming the second type), suggesting that LMG 1531^{T}, LMG 1530, LMG 27543 and LMG 28092 are at least two different strains.

Random amplified polymorphic DNA (RAPD) analysis was performed

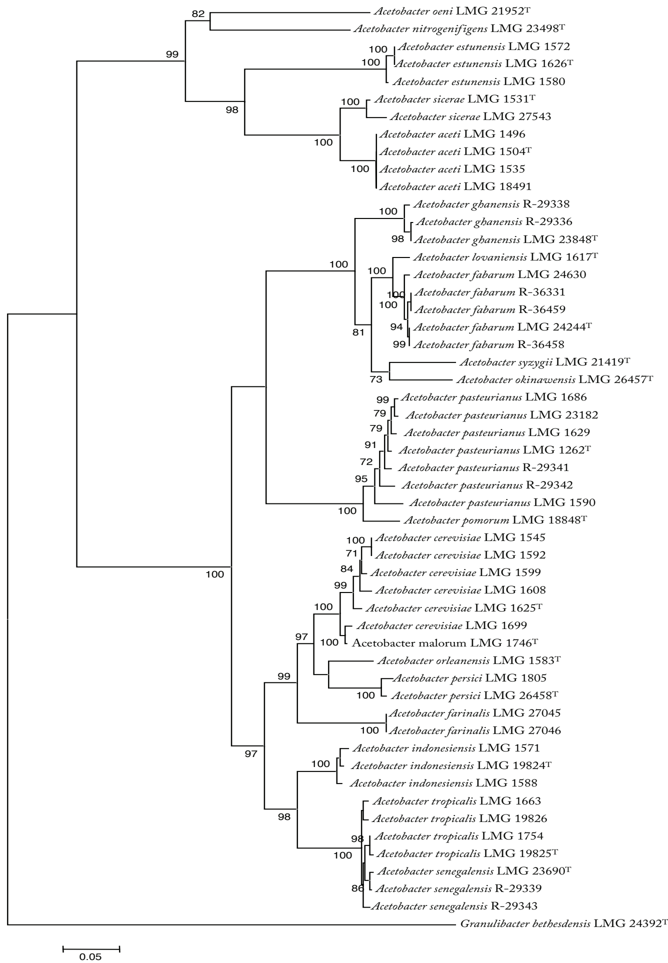


Figure 4.3. Maximum Likelihood tree based on concatenated sequences (1614 bp) of 3 housekeeping gene fragments [*dnaK* (522 bp), *groEL*(528 bp) and *rpoB*(564 bp)] showing the phylogenetic position of *Acetobacter sicerae* sp. nov. within the genus *Acetobacter*. The type strain of *Granulibacter bethesdensis* was used as outgroup. Numbers at branching points are percentage bootstrap values based on 1000 replications. The sequence accession numbers for *dnaK*, *groEL* and *rpoB* gene sequences are provided in Table S4.1. Bar, 5 % sequence divergence.

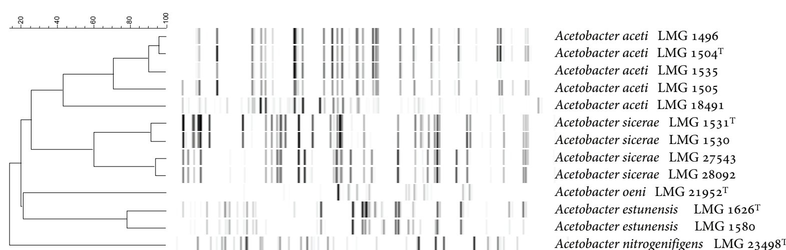


Figure 4.4. AFLP™ fingerprints of *Acetobacter sicerae* sp. nov. and their closest phylogenetic relatives. The dendrogram was derived from unweighted pair group cluster analysis (UPGMA) of the fingerprints with levels of linkage expressed as Dice similarity coefficients.

on strains LMG 1531^T, LMG 1530, LMG 27543 and LMG 28092 as previously described (Williams *et al.*, 1990). Using primer RAPD-270, three different band patterns were obtained. Those of LMG 1530 and its mutant LMG 1531^T showed a few differences, while no clear differences were found for LMG 27543 and LMG 28092 (Figure 4.5). The latter isolates were obtained from the same sample at the same time and are most probably re-isolates of the same strain.

DNA-DNA hybridizations were performed between strains LMG 1531^T and LMG 27543, and towards their nearest neighbours, *A. aceti* and *A. nitrogenifigens*, to confirm the single novel species status of both strains. Genomic DNA was extracted at large scale using the method described previously (Cleenwerck *et al.*, 2002). DNA-DNA hybridisations were performed at 46 ℃ using a modification (Goris *et al.*, 1998) of the microwell plate method (Ezaki *et al.*, 1989). Reciprocal reactions (AxB and BxA) were performed for each DNA pair. A high DNA-DNA relatedness was found between strains LMG 27543 and LMG 1531^T (88 %), and a low relatedness ($<$ 70 %) towards the type strains of *A. aceti* LMG 1504^T ($<$ 53 %) and *A. nitrogenifigens* LMG 23498^T ($<$ 15 %) (Table S4.2). The DNA-DNA hybridisation data therefore confirmed that strains LMG 1531^T and LMG 27543 constituted a single

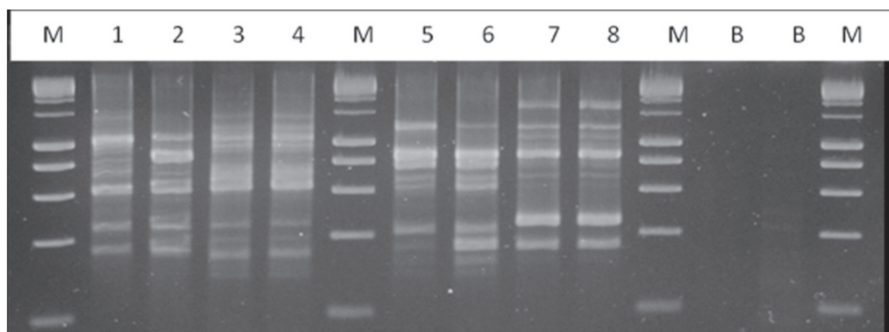


Figure 4.5. RAPD fingerprints of *Acetobacter sicerae* sp. nov. strains LMG 1530, LMG 1531^T, LMG 27543 and LMG 28092.

novel species. The DNA G+C content of strains LMG 1531^T and LMG 27543 was 58.3 and 56.7 mol %, respectively, which is consistent with DNA G+C contents of members of the genus *Acetobacter* (Cleenwerck *et al.*, 2008; Iino *et al.*, 2012b).

The whole cell fatty acid methyl ester (FAME) composition was determined for strains LMG 1531^T and LMG 27543 and *A. aceti* LMG 1504^T using an Agilent Technologies 6890N gas chromatograph (Santa Clara, CA, USA). Cultivation of the strains, fatty acid, extraction and analysis of the fatty acid methyl esters were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10 (MIDI). Fatty acids were extracted from cultures grown in AAM for 48 h at 28 ℃ under aerobic conditions. The peaks of the profiles were identified using the TSBA50 identification library version 5.0 (MIDI, Hewlett Packard, Newark, DE, USA). The predominant fatty acid was $C_{18:1}\omega 7c$ (54.2 %-58.3 %), while the following fatty acids were present in lower percentages (above 1 %): $C_{16:0}$ (11.2 %-11.65 %), $C_{14:0}$2-OH (10.56 %-12.85 %), $C_{16:0}$2-OH (4.33 %-5.77 %), $C_{18:0}$ (3.71 %-4.14 %), $C_{16:0}$3-OH (3.07 %-3.44 %), $C_{18:0}$3-OH (3.07 %-3.23 %) and $C_{14:0}$ (1.93 %-2.72 %) (Table 4.1). The FAME data were consistent with those reported for the known *Acetobacter* species by Spitaels (Spitaels *et al.*, 2014b), generated using the same method from cultures also

grown on AAM at 28 °C under aerobic conditions, for 24 to 72 hours, depending on the strain. The analysis of respiratory quinones of LMG 1531^{T} was performed as described previously (Vaz-Moreira *et al.*, 2007) using the method of Tindall (Tindall, 1988). The major ubiquinone present was Q-9, which was consistent with previous studies showing that Q-9 ubiquinone enables the genus *Acetobacter* to be differentiated from the other genera (Yamada and Yukphan, 2008).

Strains LMG 1531^{T} and LMG 27543 were subjected to phenotypic tests to identify characteristics enabling their differentiation from the established *Acetobacter* species, using methods described previously (Cleenwerck *et al.*, 2002; Cleenwerck *et al.*, 2007). The production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid from D-glucose was determined as reported by (Spitaels *et al.*, 2014b). The type strains of *A. aceti*, *A. nitrogenifigens* and *Acetobacter oeni* were investigated when appropriate, concurrently with strain LMG 1531^{T} and LMG 27543. Strains LMG 1531^{T} and LMG 27543 could be differentiated from their nearest neighbour species based on their ability to grow on D-fructose and D-sorbitol as the sole carbon source; their ability to grow on yeast extract with 30 % D-glucose and on standard medium [5 %, w/v, D-glucose; 0.5 %, w/v, yeast extract (Oxoid)] with a pH of 3.6 or containing 1 % NaCl; and their weak acid production from D-arabinose (Table 4.2 and Table S4.3). The production of cellulose was examined by boiling cell pellicle in 5 % NaOH for 2 hours (Navarro *et al.*, 1999). Only strain (LMG 1530) produced a cellulose pellicle.

Fatty acid	$C_{14:0}$	$C_{14:0}$ 2-OH	$C_{16:0}$	$C_{16:0}$ 2-OH	$C_{16:0}$ 3-OH	$C_{18:0}$	$C_{18:0}$ 3-OH	$C_{18:1}$ ω7c	$C_{19:0}$ cycloω8c
1	**1.9**	**10.6**	**11.7**	**4.3**	**3.1**	**4.1**	**3.1**	**58.3**	**-**
2	2.7	12.9	11.2	5.8	3.4	3.7	3.2	54.2	-
3	4.3	21.8	11.7	14.5	3.7	-	-	40.4	-
4	-	16.2	10.3	23.1	5.5	-	2.3	33.4	1.8
5	1.2	8.7	9.8	10.6	4.4	4.4	8.0	48.1	-
6	3.6	5.4	11.6	4.0	2.6	4.2	3.3	61.7	1.9
7	6.3	15.4	8.2	12.5	7.6	-	4.8	41.4	-
8	3.9	16.5	8.2	13.0	6.9	1.4	3.0	42.8	-
9	2.8	14.1	8.0	13.2	7.4	1.9	6.1	33.8	-
10	1.5	9.2	10.0	8.3	4.5	2.5	3.7	52.8	-
11	1.9	6.0	11.0	10.2	4.6	4.1	4.0	53.8	1.0
12	5.5	9.4	11.4	13.3	3.7	3.5	1.6	46.3	2.6
13	6.0	2.1	8.5	10.0	3.1	2.6	1.0	61.5	3.1
14	4.5	3.8	9.3	9.6	2.6	3.5	1.5	61.4	2.0
15	6.1	2.5	10.7	7.9	2.4	3.3	1.2	60.9	2.2
16	4.6	2.6	9.5	10.3	2.9	4.4	1.5	59.3	2.2
17	6.0	1.1	9.0	9.1	2.2	2.0	0.9	65.4	1.8
18	2.0	9.2	9.7	10.2	2.2	2.2	0.5	60.0	2.6
19	0.9	3.9	11.1	5.4	2.2	5.9	3.6	63.0	1.0
20	1.0	2.3	11.0	4.5	3.8	5.1	4.1	62.1	1.9
21	1.2	5.1	11.2	7.4	2.6	4.0	1.8	64.6	-
22	1.1	4.4	11.4	6.6	2.3	3.9	1.9	64.8	-
23	-	5.2	10.7	6.4	2.6	4.7	3.1	61.5	-
24	2.2	6.5	10.3	6.3	4.7	3.1	1.6	61.9	1.6
25	-	3.2	13.0	8.2	2.4	6.1	3.0	58.1	3.1
26	1.1	1.2	10.5	8.8	1.9	8.3	1.2	59.3	2.1

Table 4.1. Cellular fatty acid contents (%) of *Acetobacter sicerae* sp. nov. (data in bold) and all *Acetobacter* type strains.

1. *A. sicerae* (LMG 1531^{T}); 2. *A. sicerae* (LMG 27543); 3. *A. aceti* (LMG 1504^{T}); 4. *A. nitrogenifigens* (LMG 23498^{T}); 5. *A. oeni* (LMG 21952^{T}); 6. *A. estunensis* (LMG 1626^{T}); 7. *A. pomorum* (LMG 18848^{T}); 8. *A. pasteurianus* (LMG 1262^{T}); 9. *A. senegalensis* (LMG 23690^{T}) ; 10. *A. tropicalis* (LMG 19825^{T}); 11. *A. indonesiensis* (LMG 19824^{T}); 12. *A. papayae* (LMG 26456^{T}); 13. *A. fabarum* (LMG 24244^{T}); 14. *A. ghanensis* (LMG 23848^{T}); 15. *A. syzygii* (LMG 21419^{T}); 16. *A. okinawensis* (LMG 26457^{T}); 17. *A. lovaniensis* (LMG 1617^{T}); 18. *A. peroxydans* (LMG 1635^{T}); 19. *A. cerevisiae* (LMG 1625^{T}); 20. *A. cibinongensis* (LMG 21418^{T}); 21. *A. orleanensis* (LMG 1583^{T}); 22. *A. persicus* (LMG 26458^{T}); 23. *A. malorum* (LMG 1746^{T}); 24. *A. orientalis* (LMG 21417^{T}); 25. *A. farinalis* (LMG 26772^{T}) ; 26. *A. lambici* (LMG 27439^{T}). -, not detectable or trace amount ($<$1 %). Data of *A. sicerae* LMG 1531^{T}, LMG 27543, and *A. aceti* LMG 1504^{T} were generated in the frame of this study. Other data were taken from (Spitaels *et al.*, 2014b). Cultivation conditions prior to fatty acid extraction were identical for all strains, except for the duration of cultivation that varied from 24h to 72h depending on the strain.

Table 4.2. Differential characteristics between *Acetobacter sicerae* sp. nov. (LMG 1531^T and LMG 27543) and the phylogenetic closest *Acetobacter* species, *A. aceti* (4 strains, including LMG 1504^T), *A. nitrogenifigens* LMG 23498^T, *A. oeni* LMG 21952^T and *A. estunensis* LMG 1626^T. Data were obtained in this study, unless indicated otherwise. +: positive, -: negative, w: weak positive, v: variable (the result of the type strain is given between brackets), SM, standard medium. Data were obtained in this study, unless indicated otherwise.

Feature	*A. sicerae*	*A. aceti*	*A. nitrogenifigens*	*A. oeni*	*A. estunensis*
Formation from D-glucose:					
5-keto-D-gluconic acid	+	+[a]	+[b]	+[a]	-[a]
2-keto-D-gluconic acid	+	+[a]	-[b]	-[a]	+[a]
Growth in ammonium with ethanol	+	+	+	-	+
Growth in 10 % ethanol	-	-	+	+	-
Growth on YE + 30 % D-glucose	+	-	+	-	-
Growth on carbon sources:					
D-fructose	+	-	+	+	+
D-sorbitol	+	v (-)	-	-	+
Acid production from					
D-arabinose	w[c]	v (+)	+	+	+
Growth on SM with 1 % NaCl	+	-	-	+	-
Growth on SM at pH3.6	+	v (w)	-	+	w

[a] Data taken from Cleenwerck *et al.* (2008).
[b] Data taken from Spitaels *et al.* (2014).
[c] Color change was observed, with pH range between 5.98 6.05, while + was described as color change and a pH measurement lower than 5.9 (Gosselé *et al.*, 1983b).

4.3. Conclusion

In conclusion, the results presented above demonstrate that strains LMG 1531^T, LMG 1530, LMG 27543 and LMG 28092 represent a single novel species that can be differentiated genotypically and phenotypically from the currently established species of the genus *Acetobacter*. Therefore, we propose to classify them as the novel species, *Acetobacter sicerae* sp. nov., with strain LMG 1531^T (= NCIMB 8941^T) as the type strain.

Description of *Acetobacter sicerae* sp. nov. *Acetobacter sicerae* (*si.c'rae*, L. gen. n. *sicerae*, of a fermented liquor, intended to mean of cider)

Cells are Gram-negative, motile, coccoid rods, approximately 1 μm wide and 1.5–2.5 μm long. Cells occur singly or in pairs. Catalase and oxidase activity is present. On LMG medium 404 agar (`http://bccm.belspo.be/db/media_search_form.php`), colonies are round, smooth, beige and slightly raised, with a diameter of approximately 1 mm after 2 days of incubation. Able to produce 2-keto-D- gluconic and 5-keto-D-gluconic acid from D-glucose. Able to grow on D-fructose, D-sorbitol and glycerol as single carbon sources, but not on maltose or methanol. Able to grow on ammonium as sole nitrogen source with ethanol as carbon source. Able to grow on yeast extract containing 30 % D-glucose and on standard medium with a pH of 3.6 or containing 1 % NaCl. Able to produce acid from D-arabinose weakly. The predominant fatty acid is $C_{18:1}\omega$ 7c; other fatty acids in significant amounts are $C_{14:0}$2-OH, $C_{16:0}$, $C_{16:0}$2-OH and $C_{18:0}$, The DNA G+C content of the type strain is 58.3 %.

The type strain, LMG 1531^T (= NCIMB 8941^T), is a non-cellulose-producing mutant from the peritrichous flagellated strain LMG 1530, which was isolated by J.Carr from cider (Shimwell and Carr, 1958).

4.4. Acknowledgements

Leilei Li has a PhD grant of Chinese Scholarship Council and Ghent University Co-Funding. The MLSA work was supported by funds

from the European Community's Seventh Framework Programme (FP7, 2007-2013), Research Infrastructures action, under the grant agreement No. FP7-228310 (EMbaRC project). The BCCM/LMG Bacteria Collection is supported by the Federal Public Planning Service-Science Policy, Belgium. Katrien Engelbeen and Marjan De Wachter are acknowledged for help with the DNA-DNA hybridizations and performance of the AFLP data, respectively.

4.5. Supplementary materials

The Genbank/EMBL accession numbers for the 16S rRNA, NCBI accession numbers for the *dnaK*, *groEL* and *rpoB* gene sequences generated in this study are HG424424, HG424425, HG424426, KJ147511 and KJ147512 for the 16S rRNA gene sequences; KF537390–KFKF537407, KF537409–KF537428 and KF537430–KF537432 for *dnaK* gene sequences; KF537433–KF537482 for *groEL* gene sequences and KF537483–KF537492, KF537494 and KF537496–KF537531 for *rpoB* gene sequences.

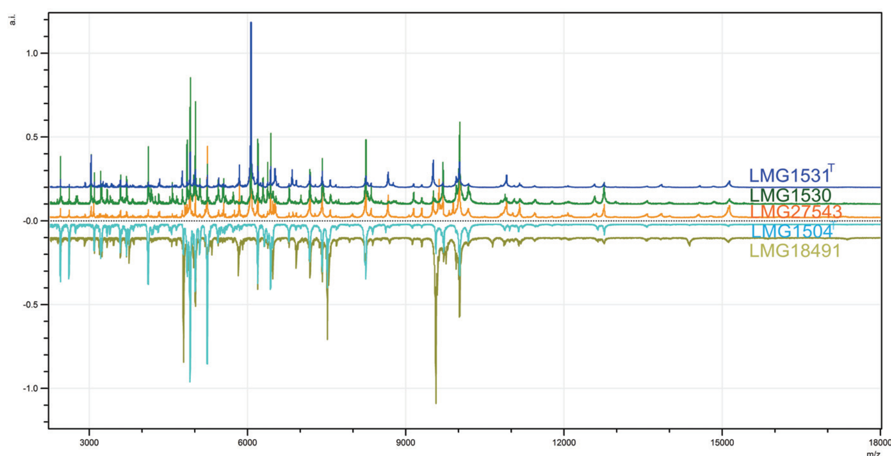


Figure S4.1. Comparison of MALDI-TOF MS profiles of *A. sicerae* LMG 1531^{T}, LMG 1530 and LMG 27543, and *A. aceti* LMG 1504^{T} and LMG 18491 using the mMass 5.1.0 software.

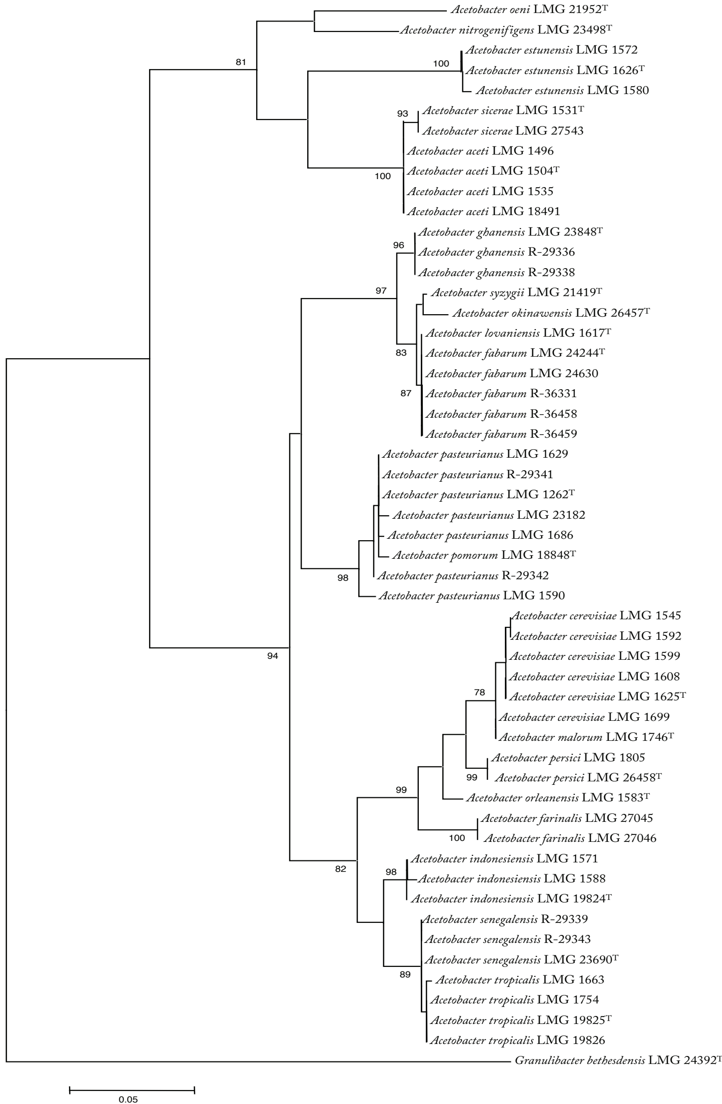


Figure S4.2. Maximum Likelihood tree based on concatenated amino acids sequences of 3 housekeeping gene fragments (538 aa). The type strain of *Granulibacter bethesdensis* was used as outgroup. Numbers at branching points are percentage bootstrap values based on 1000 replications. Bar, 5 % sequence divergence.

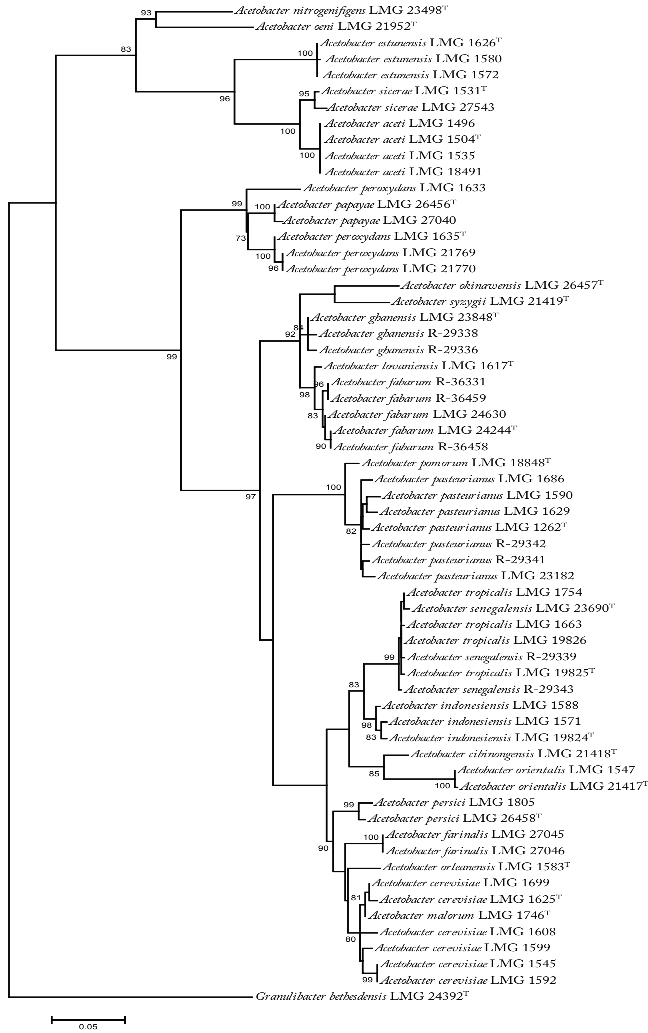


Figure S4.3. Maximum Likelihood tree of all established *Acetobacter* species based on partial *groEL* gene sequences (528 bp). The type strain of *Granulibacter bethesdensis* was used as outgroup. Numbers at branching points are percentage bootstrap values based on 1000 replications. The sequence accession numbers are provided in Table S4.1. Bar, 5 % sequence divergence.

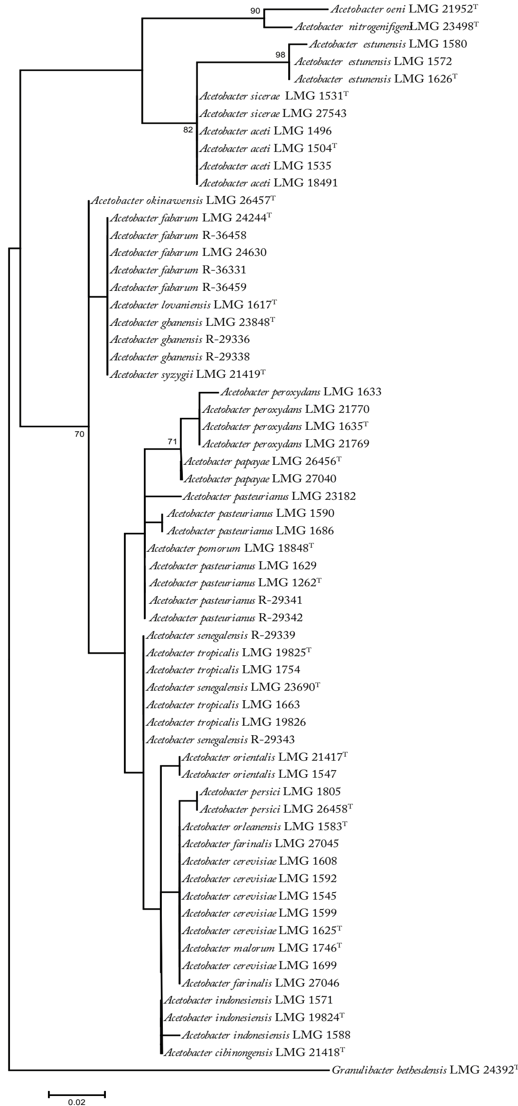


Figure S4.4. Maximum Likelihood tree of all established *Acetobacter* species based on partial *groEL* gene amino acid sequences (176 aa). The type strain of *Granulibacter bethesdensis* was used as outgroup. Numbers at branching points are percentage bootstrap values based on 1000 replications. Bar, 2 % sequence divergence.

Table S4.1. Accession numbers of housekeeping genes sequences of *dnaK*, *groEL* and *rpoB*.

Taxon	LMG number	Genes		
		dnaK	*groEL*	*rpoB*
Acetobacter aceti	LMG1504^{T}	FN421342	FN421343	FN421344
Acetobacter aceti	LMG1496	KF537398	KF537440	KF537485
Acetobacter aceti	LMG1535	KF537399	KF537441	KF537486
Acetobacter aceti	LMG18491	KF537397	KF537442	KF537487
Acetobacter cerevisiae	LMG1545	KF537427	KF537479	KF537488
Acetobacter cerevisiae	LMG1592	KF537428	KF537480	KF537489
Acetobacter cerevisiae	LMG1599	KF537425	KF537481	KF537490
Acetobacter cerevisiae	LMG1608	KF537426	KF537482	KF537491
Acetobacter cerevisiae	LMG1625^{T}	KF537424	KF537477	KF537492
Acetobacter cerevisiae	LMG1699	KF537430	KF537478	KF537494
Acetobacter cibinongensis	LMG21418^{T}		KF537458	
Acetobacter estunensis	LMG1572	KF537393	KF537436	KF537496
Acetobacter estunensis	LMG1580	KF537392	KF537435	KF537497
Acetobacter estunensis	LMG1626^{T}	KF537394	KF537437	KF537498
Acetobacter fabarum	LMG24630	KF537432	KF537457	KF537483
Acetobacter fabarum	LMG24244^{T}	HG329536	HG329548	HG329560
Acetobacter fabarum	R-36331	HG329540	HG329552	HG329564
Acetobacter fabarum	R-36458	HG329541	HG329553	HG329565
Acetobacter fabarum	R-36459	HG329542	HG329554	HG329566
Acetobacter farinalis	LMG27045	KF537416	KF537474	KF537499
Acetobacter farinalis	LMG27046	KF537417	KF537475	KF537500
Acetobacter ghanensis	LMG23848^{T}	HG329535	HG329547	HG329559
Acetobacter ghanensis	R-29336	HG329538	HG329550	HG329562
Acetobacter ghanensis	R-29338	HG329539	HG329551	HG329563
Acetobacter indonesiensis	LMG1571	KF537419	KF537461	KF537501
Acetobacter indonesiensis	LMG1588	KF537418	KF537462	KF537502
Acetobacter indonesiensis	LMG19824^{T}	KF537420	KF537463	KF537503
Acetobacter lovaniensis	LMG1617^{T}	HG329533	HG329545	HG329557
Acetobacter malorum	LMG1746^{T}	KF537431	KF537476	KF537504
Acetobacter nitrogenifigens	LMG23498^{T}	KF537390	KF537433	KF537505
Acetobacter oeni	LMG21952^{T}	KF537391	KF537434	KF537506
Acetobacter okinawensis	LMG26457^{T}	HG329537	HG329549	HG329561
Acetobacter orientalis	LMG21417^{T}		KF537460	
Acetobacter orientalis	LMG1547		KF537459	
Acetobacter orleanensis	LMG1583^{T}	KF537421	KF537473	KF537507
Acetobacter papayae	LMG26456^{T}		KF537444	KF537509

Table S4.1. Accession numbers of housekeeping genes sequences of *dnaK*, *groEL* and *rpoB*.

			Genes	
Taxon	LMG number	*dnaK*	*groEL*	*rpoB*
Acetobacter papayae	LMG27040		KF537443	KF537508
Acetobacter pasteurianus	LMG1262^T	KF537405	KF537450	KF537510
Acetobacter pasteurianus	LMG1590	KF537407	KF537451	KF537516
Acetobacter pasteurianus	LMG1629	KF537404	KF537452	KF537511
Acetobacter pasteurianus	LMG1686	KF537402	KF537453	KF537512
Acetobacter pasteurianus	LMG23182	KF537403	KF537454	KF537513
Acetobacter pasteurianus	R-29341	KF537406	KF537455	KF537514
Acetobacter pasteurianus	R-29342	KF537401	KF537456	KF537515
Acetobacter peroxydans	LMG21769		KF537447	KF537518
Acetobacter peroxydans	LMG21770		KF537448	KF537530
Acetobacter peroxydans	LMG1635^T		KF537446	KF537519
Acetobacter peroxydans	LMG1633		KF537445	KF537517
Acetobacter persicus	LMG1805	KF537422	KF537472	KF537484
Acetobacter persicus	LMG26458^T	KF537423	KF537471	KF537531
Acetobacter pomorum	LMG18848^T	KF537400	KF537449	KF537520
Acetobacter senegalensis	LMG23690^T	KF537415	KF537464	KF537521
Acetobacter senegalensis	R-29339	KF537414	KF537465	KF537522
Acetobacter senegalensis	R-29343	KF537413	KF537466	KF537515
Acetobacter sicerae	LMG1531^T	KF537395	KF537438	KF537524
Acetobacter sicerae	LMG27543	KF537396	KF537439	KF537525
Acetobacter syzygii	LMG 21419^T	HG329534	HG329546	HG329558
Acetobacter tropicalis	LMG1663	KF537410	KF537467	KF537529
Acetobacter tropicalis	LMG1754	KF537412	KF537468	KF537526
Acetobacter tropicalis	LMG19825^T	KF537411	KF537469	KF537527
Acetobacter tropicalis	LMG19826	KF537409	KF537470	KF537528

Table S4.2. DNA-DNA relatedness of *A. sicerae* and the type strains of its closest phylogenetic relatives.

	1	2	3	4
1. *A. sicerae* LMG 1531^T	100 %			
2. *A. sicerae* LMG 27543	87 %	100 %		
3. *A. aceti* LMG 1504^T	53 %	50 %	100 %	
4. *A. nitrogenifigens* LMG 23498^T	9 %	12 %	9 %	100 %

Table S4.3. Characteristics between *Acetobacter sicerae* and all established *Acetobacter* species.

Taxa are listed as: 1. *A. sicerae* sp. nov. (2 strains); 2. *A. aceti* (4 strains); 3. *A. nitrogenifigens* LMG 23498^T; 4. *A. oeni* LMG 21952^T; 5. *A. estunensis* (3 strains); 6. *A. okinawensis* (7 strains); 7. *A. ghanensis* (3 strains); 8. *A. syzygii* LMG 21419^T; 9. *A. fabarum* (4 strains); 10. *A. lovaniensis* LMG 1617^T; 11. *A. peroxydans* (2 strains); 12. *A. cerevisiae* (4 strains); 13. *A. cibinongensis* LMG 21418^T; 14. *A. orleanensis* (4 strains); 15. *A. persici* (2 strains); 16. *A. malorum* LMG 1746^T; 17. *A. orientalis* LMG 21417^T; 18. *A. farinalis* (3 strains); 19. *A. tropicalis* (2 strains); 20. *A. indonesiensis* (2 strains); 21. *A. papayae* (2 strains); 22. *A. pomorum* LMG 18848^T; 23. *A. pasteurianus* (7 strains); 24. *A. senegalensis* (3 strains); 25. *A. lambici* (4 strains).The type strain is included for all taxa. +: positive, -: negative, w: weak positive, v: variable (the result of the type strain is given between brackets), nr: not reported. Data for taxon 1 were obtained in this study, unless indicated otherwise: data for taxa 2, 4, 5 and 7–22 were taken from Cleenwerck *et al*(2008); data for taxa 3, 6 and 24 were taken from Iino *et al.* (2012); data for taxon 23 were taken from Tanasupawat *et al.* (2011b); data for taxon 25 were taken from Spitaels *et al*(2014b).

Table S4.3. Differential characteristics between *Acetobacter sicerae* and all established *Acetobacter* species.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Formation from D-glucose:																									
5-keto-D-gluconic acid	+	+	+[a]	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
2-keto-D-gluconic acid	+	+	-[a]	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-	v(-)[a]	+	-
Growth in ammonium with ethanol	+	+[b]	+[b]	-[b]	+[b]	-	-	-	v(+)	+	+	-	w	-	-	-	-	w	-	-	-	-	-	+	-
Growth in 10% ethanol	-	-[b]	+[b]	+[b]	-[b]	v(+)	v	-	v(-)	-	-		-	-	+	+	-	-	-	-	+	-	+	+	-
Growth on YE+30% D-glucose	+	-[b]	+[b]	-[b]	-[b]	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	v(-)	+	-
Growth on carbon sources:																									
Glycerol	+	+	+	+	v (nr)	+[a]	w	+	+	+	-	+	+	+	+[a]	+	+	+	+	+	w[a]	+	v (nr)	+	-
Methanol	-	-	-	-	-	+[a]	-	-	+	+	-	-	-	-	nr	+	w	-	-	-	nr	-	-	-	-
Acid production from																									
L-arabinose	+	+[b]	nr	nr	nr	-	+	v (-)[a]	-	v(+)	-	-	nr	v(-)	+	-	nr	+	nr	nr	+	nr	+	nr	-
D-galactose	+	+[b]	+[b]	+[b]	+[b]	-	-	-	-	v(+)	-	+	nr	v(-)	+	+	nr	+	nr	nr	+	nr	nr	nr	-
Catalase	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-[a]	+	+	+v +	+v +	+		
G+C content of DNA (mol%)	56.7-58.3	56.9-58.3	64.1	58.1	59.2-60.2	59.2-59.4	56.9-57.3	54.3-55.4	56.8-58.0	57.1-58.9	59.7-60.7	56.0-57.6	53.8-54.5	55.7-58.1	58.7-58.9	57.2	52.0-52.8	56.3-56.5	55.6-56.2	54.0-54.2	60.5-60.7	52.1	53.2-54.3	55.6-56.0	56.2

[a] data taken from Spitaels *et al.* (2014).
[b] data obtained in the present study.

5

Bombella intestini gen. nov., sp. nov., an acetic acid bacterium isolated from bumble bee crop

Leilei Li, Jessy Praet, Wim Borremans, Olga C. Nunes, Célia M. Manaia, Ilse Cleenwerck, Ivan Meeus, Guy Smagghe, Luc De Vuyst, Peter Vandamme

Redrafted from: International Journal of Systematics and Evolutionary Microbiology, 2015, 65(1), pp 267–273, doi: 10.1099/ijs.0.068049-0

Summary

In the frame of a bumble bee 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 LMG 28161^T was determined and showed the highest pairwise similarity to *Saccharibacter floricola* (96.0 %) and *Acetobacter estunensis* (94.1 %), which corresponded with genus level divergence in the *Acetobacteraceae* family. 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 data obtained confirmed that this isolate is best classified into a novel genus in the family *Acetobacteraceae*. Its DNA G+C content was 54.9 mol %. The fatty acid compositions of R-52486 and LMG 28161^T were similar to those of established AAB species [with $C_{18:1}\omega 7c$ (43.3 %) as 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 lack of ability 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-gluconic acid, but not 5-keto-D-gluconic acid from D-glucose. Therefore, the name *Bombella intestini* is proposed for this new taxon, with LMG 28161^T (= DSM 28636^T) as the type strain.

5.1. 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, *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, *Asaia* and *Saccharibacter* species have frequently been associated with honey bees (*Apis mellifera*) (Babendreier *et al.*, 2007; Crotti *et al.*, 2009; Crotti *et al.*, 2010; Gilliam, 2010; Martinson *et al.*, 2011; Mohr and Tebbe, 2006), but only rarely with bumble bees (*Bombus* spp.) (Koch and Schmid-Hempel, 2011; Martinson *et al.*, 2011; Olofsson and Vásquez, 2009). Yet, a recent study has revealed two *Acetobacteraceae* as relevant bacteria of the *Bombus bimaculatus* gut microbiota (Cariveau *et al.*, 2014).

5.2. methods, results and discussion

In the frame of a bumble bee gut microbiota study, bumble bees were caught in the field, in Ghent, Belgium, frozen at -20 ℃ for 10 min and surface-sterilized with 2.5 % Umonium38®Master (Laboratoire Huckert's International, Brussels, Belgium) 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 ℃ 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 of suspension was added to 25 ml of both enrichment medium I (EM I) and II (EM II), as described by (Lisdiyanti *et al.*, 2003), 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 ℃ for 7 days. Enrichment media showing bacterial growth were subsequently plated onto four solid agar media, *i.e.* LMG medium 13 [2.5 %, w/v, D-mannitol; 0.5 %, w/v, yeast extract (Oxoid, Basingstoke, UK); 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 containing 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 same four solid agar media as mentioned above. All inoculated plates were incubated aerobically at 28 ℃ for 7 days. The LMG media 13 and 404 were the sole media that yielded growth. Colonies were picked up from agar media that were inoculated both directly as well as after enrichment in EM II. Isolates (Table S5.1) were dereplicated by matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS), as described by Wieme (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 (Figure S5.1). 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 honey bee gut, crop or larvae (Anderson *et al.*, 2013; Koch *et al.*, 2013; Martinson *et al.*, 2011; 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 S5.2) and which was isolated from the same bumble bee 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* (96.5 %; accession number, AB110421). This rather low nearest neighbor similarity value suggested that this isolate should be classified into a new genus of the *Acetobacteraceae* family. The 16S rRNA gene sequence of LMG 28161^T and of type strains of species of this family, representing all known genera, were aligned against the SILVA bacteria database using the Mothur pipeline (Quast *et al.*, 2013; Schloss *et al.*, 2009). Subsequently, phylogenetic trees were constructed with MEGA 6, using the neighbor-joining (NJ) and maximum-likelihood (ML) 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.*, 2013). 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 5.1).

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.*, unpublished results). A 16S-23S rRNA internal transcribed spacer (ITS) sequence and sequences of the housekeeping gene *groEL* was extracted for phylogenetic analyses, as these sequences were proven useful to refine the phylogeny of members of the family *Acetobacteraceae* (Cleenwerck *et al.*, 2002; Li *et al.*, 2014; Trček and Teuber, 2002). A *groEL* sequence was also extracted from the draft genome of *S. floricola* DSM 15669^T, available online (accession number, NZ_ARJS00000000.1). A phylogenetic analysis based on 16S-23S rRNA ITS sequences was performed using MEGA 6 software package; a phylo-

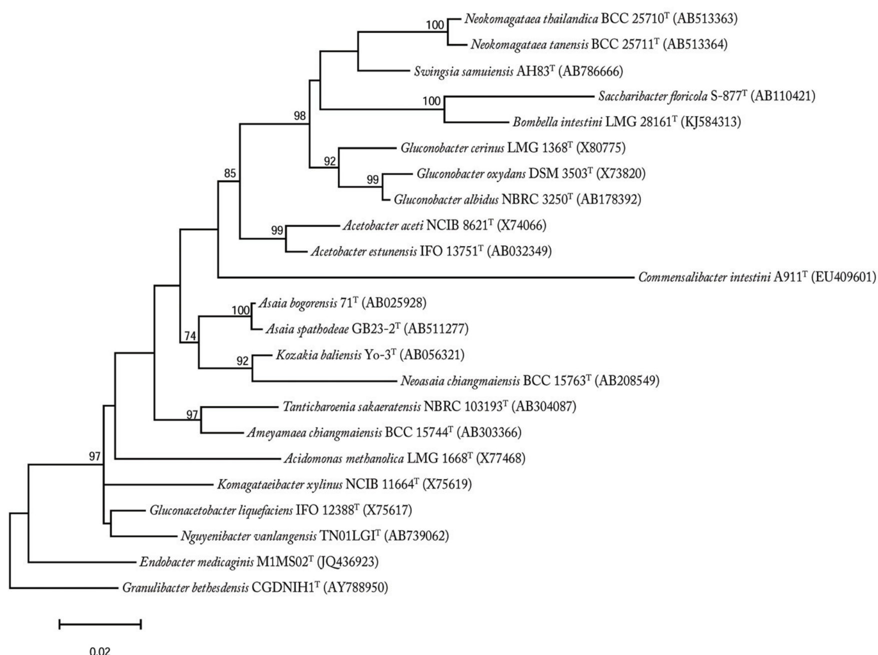


Figure 5.1. Maximum-likelihood tree based on nearly complete 16S rRNA gene sequences (1353-1431 nt), showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. within the family *Acetobacteraceae*. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 2 % sequence divergence.

genetic tree was constructed using the neighbor-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 neighbors (Figure S5.3). The phylogenetic position of isolate LMG 28161^{T}, based on partial sequences of the housekeeping gene *groEL* (720 nt, Figure S5.4), and based on the corresponding amino acid sequences (240 aa, Figure S5.5), was also determined as described before (Li *et al.*, 2014) 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 this family.

Random amplified polymorphic DNA (RAPD) analysis was performed on 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 re-isolates of the same strain (Figure S5.6).

The DNA G+C content of isolate LMG 28161^{T} was determined as described previously (Cleenwerck *et al.*, 2008) and was 54.9 mol %. This value falls within the range reported for the family *Acetobacteraceae* (Greenberg *et al.*, 2006)and is also consistent with the whole-genome shotgun sequencing data, which revealed a value of 55.0 %.

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 genera of the family *Acetobacteraceae*, using an Agilent Technologies 6890N gas chromatograph (Santa Clara, CA, USA). 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, Hewlett Packard, Newark, DE, USA), except that fatty acids were extracted from cultures grown on LMG medium 404 at 28 ℃ under aerobic conditions for 48 to 72 hours (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 $C_{18:1}\omega 7c$ (43.1 %), while the following fatty acids were present in lower percentages (above 1 %):

$C_{19:0}$*cyclo*ω8*c* (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 5.1). The fatty acid composition of LMG 28161^T was similar to that of known AAB species (with $C_{18:1}\omega 7c$ as major component), but the amounts of fatty acids, such as $C_{19:0}$*cyclo*ω8*c*, $C_{14:0}$ and $C_{14:0}$2-OH, enabled to differentiate it (Table 5.1 and Table S5.2). The analysis of respiratory quinones of LMG 28161^T was performed, as described previously (Vaz-Moreira *et al.*, 2007) using the method of Tindall (Tindall, 1988). The major ubiquinone present was Q-10, which agreed with the major ubiquinone of most other genera in the family *Acetobacteraceae*, except for *Acetobacter* species that have Q-9 as major ubiquinone; no other quinones were found.

Table 5.1. Cellular fatty acid 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
$C_{18:1}$ ω7c	43.1	29.8	51
$C_{19:0}$*cyclo*ω8*c*	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; Cleenwerck *et al.*, 2007). For microscopy and colony morphology, isolates were grown aerobically at 28 ℃ on LMG medium 404 for 48 hours. 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 acetic acid

bacteria 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 ℃ 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 micro-aerobic environment. The biochemical characteristics examined for 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 ℃ on LMG medium 404, 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.*, 2014b). 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 the genera of the family *Acetobacteraceae* by means of multiple biochemical characteristics (Table 5.2), such as their lack of ability to produce acetic acid and 5-keto-D-gluconate from ethanol and D-glucose, respectively; their ability to grow at 37 ℃ 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 *A. aceti* LMG 1504^T and *G. roseus* LMG 1418^T the published data were confirmed (Table 5.2). Therefore, a nearly complete 16S rRNA gene sequence was determined for strain LMG 23170^T (1436 nt, 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. A pairwise similarity of 100 % with 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) was found, suggesting that LMG 23170^T is a true subculture of the type strain of *S. floricola*.

5.3. Conclusion

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 neighbor species, *S. floricola*, is considerably divergent (Figure 5.1, Figure S5.3-S5.5). Its fatty acid profile is unique (Table 5.1, Table S5.2). Phenotypic characteristics that allow to distinguish this taxon from *S. floricola* are its ability to grow on LMG medium 404 at 37 ℃; 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 5.2). Therefore, the classification of this taxon into a novel species of a novel genus is warranted. We propose the name *Bombella intestini* gen. nov., sp. nov., with strain LMG 28161^T (= DSM 28636^T) as the type strain.

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

Cells are Gram-negative, straight rods. Catalase activity is present, but no oxidase activity. The predominant fatty acid is $C_{18:1}\omega 7c$; 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 honey bees and bumble bees, 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 strain LMG 28161^T 16S rRNA gene sequence suggests that the occurrence of

Table 5.2. Characteristics that differentiate *Bombella* gen. nov. from its phylogenetic 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 general had Q-10 as the major ubiquinone. +: positive, -: negative, w: weak positive, v: variable, ND: not determined.°: data obtained in the present study; * data obtained in the present study not corresponding to published data.

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	**+**	-	-	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 ammonium 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

members of this taxon is not limited to bumble bees.

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)], colonies are round, smooth, brownish and slightly raised, with a diameter of approximately 1–2 mm after 3 days of incubation. Ethanol is not oxidized to acetic acid. Production of 2-keto-D-gluconate from D-glucose, but no 5-keto-D-gluconate. Production of acid from sucrose, D-fructose, D-glucose, D-mannitol and L-arabinose (weakly). Growth in the presence of 30 % (w/v) D-glucose. Growth at 37 ℃ on LMG medium 404. No growth with ammonium as the sole nitrogen source. The DNA G+C content of the type strain is 54.9 mol %. The type strain LMG 28161^{T} (= DSM 28636^{T} = R-52487^{T}) was isolated from the crop of a bumble bee (*Bombus lapidarius*) in 2013 in Ghent, Belgium.

5.4. Acknowledgements

Leilei Li 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.

5.5. Supplementary materials

Table S5.1. Isolates from bumble bee gut samples and their identifications. (*Bombus* spp. were determined by color pattern. *B. terrestris* and *B. locorum* were 4 similar in color pattern thus could not be differentiated).

Sample no.	Bumble bee 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	Closes to *Fructobacillus tropaeoli* sequence similarity 99 %	EM II + M13	nearly complete 16S rRNA gene sequencing (1518 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 (1513 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 %	M14/M404	MALDI-TOF MS/nearly complete 16S rRNA gene sequencing

	$C_{18:1}\omega7c$	$C_{19:0}$*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	-	-	-	-

Table S5.2. 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 24037^{T}; 5. *Ameyamaea chiangmaiensis* LMG 27010^{T}; 6. *Asaia bogorensis* LMG 21650^{T}; 7. "*Commensalibacter intestini*" LMG 27436^{T}; 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.

Figure S5.1. MALDI-TOF MS identification of bumble bee isolates as *Asaia astilbis* 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.

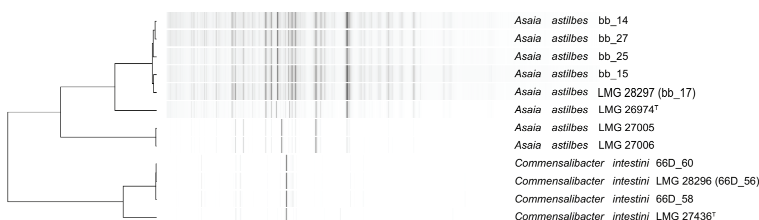


Figure S5.2. Comparison of MALDI-TOF MS profiles of *Bombella intestini* gen. nov., sp. nov. LMG 28161^{T} and R-52486, and *Saccharibacter floricola* LMG 23170^{T} using the mMass 5.1.0 software (Strohalm *et al.*, 2010). The isolates LMG 28161^{T} and R-52486 showed an identical mass spectrum.

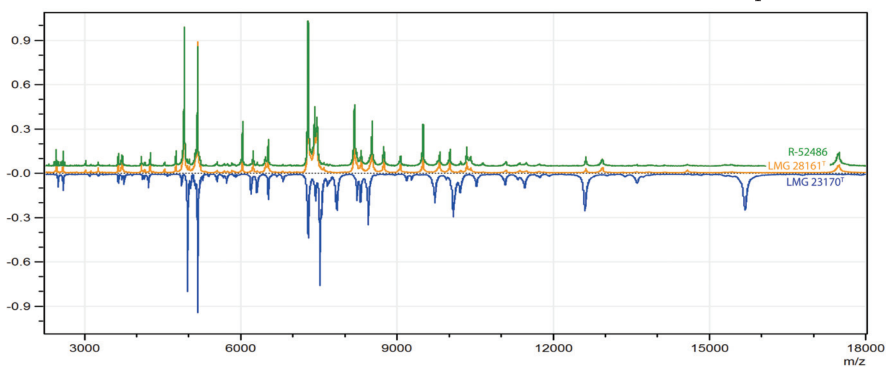


Figure S5.3. Neighbour-joining tree based on 16S-23S rRNA gene internal transcribed spacer (ITS) sequences (560 nt of LMG 28161^{T}), showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. to its close relatives. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 5 % sequence divergence.

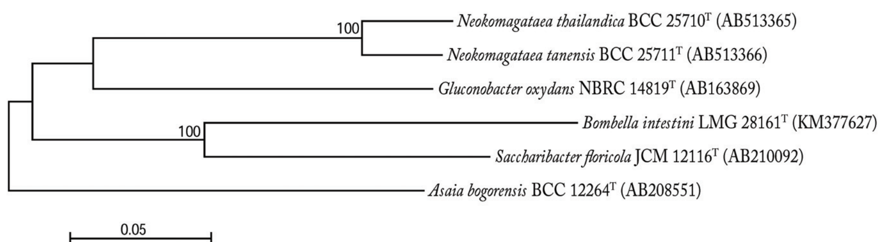


Figure S5.4. Maximum-likelihood tree based on partial housekeeping gene sequences *groEL* (720 nt), showing the phylogenetic position of *Bombella intestini* gen. nov., sp. nov. to its close relatives. The nucleotide substitution model TN93+G was chosen. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 5 % sequence divergence.

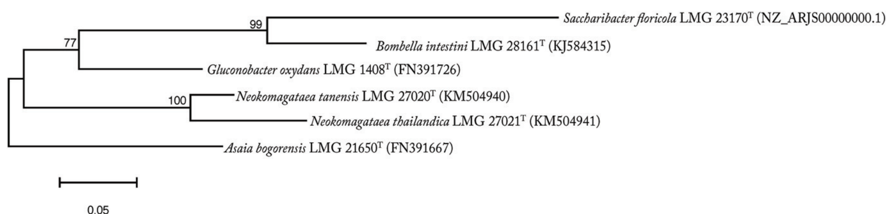


Figure S5.5. Maximum-likelihood tree based on amino acid sequences of housekeeping gene *groEL* fragments (240 aa). The amino acid substitution model LG+G was chosen. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 2 % sequence divergence.

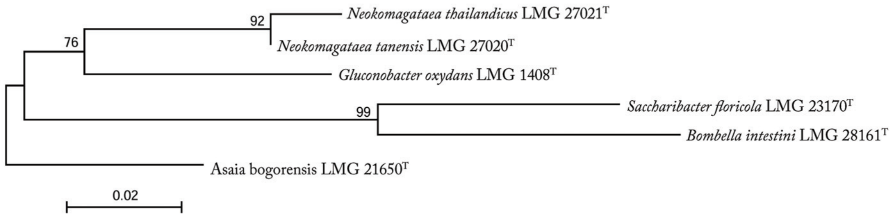
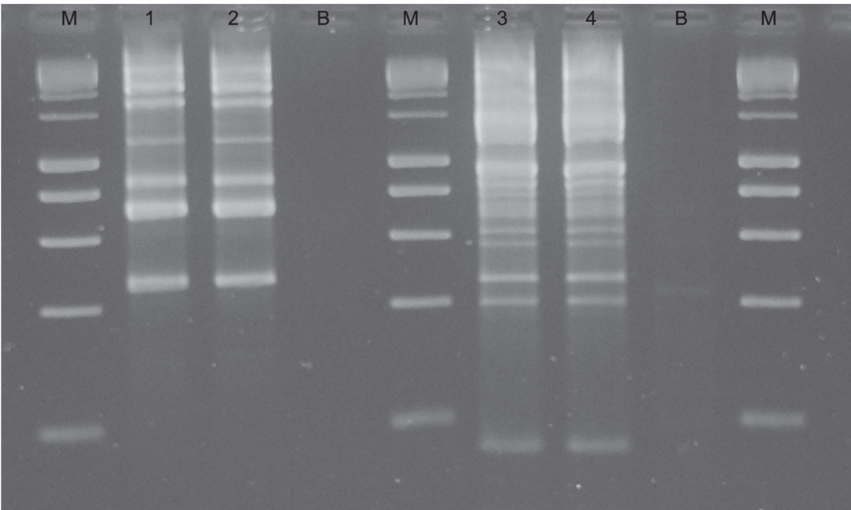


Figure S5.6. RAPD fingerprints of *Bombella intestini* gen. nov., sp. nov. R-52486 and LMG 28161^T.
Lanes 1, 2 = RAPD patterns obtained using primer RAPD-270 (5'-TGCGCGCGGG-3') of R-52486 and LMG 28161^T, respectively. Lanes 3, 4 = RAPD patterns obtained using primer RAPD-272 (5'-AGCGGGCCAA-3') of R-52486 and LMG 28161^T, respectively. Lane M, reference marker. Lane B, blank.



6

Whole genome sequence analysis of *Bombella intestini* LMG 28161^{T}, a novel acetic acid bacterium isolated from the crop of a red-tailed bumble bee, *Bombus lapidarius*

Leilei Li, Koen Illeghems, Simon Van Kerrebroeck, Wim Borremans, Ilse Cleenwerck, Guy Smagghe, Luc De Vuyst, Peter Vandamme

Summary

Bombella intestini LMG 28161^T was recently isolated from the crop of a red-tailed bumble bee (*Bombus lapidarus*) and shows 97–99 % pairwise 16S rRNA gene sequence similarity to the so-called Alpha-2.2 phylum within the family *Acetobacteraceae*. Alpha-2.2 bacteria were repeatedly detected in gut samples of honey bees (*Apis* spp.) and bumble bees (*Bombus* spp.) in the course of several metagenomic studies. *In vitro* experiments demonstrated that *B. intestini* LMG 28161^T was able to oxidize sucrose, D-glucose, D-fructose and D-mannitol, but not ethanol, and to produce 2-keto-D-gluconic acid under both aerobic and micro-aerobic conditions. The whole-genome sequence of this strain was determined to investigate the molecular mechanisms underlying its metabolic capabilities. Based on an assembled draft genome sequence of 2.03 Mb, metabolic pathways of sugars and sugar derivatives were reconstructed, which also revealed why this organism is not able to oxidize ethanol to acetic acid. Finally, several ABC transporters and type IV secretion systems that might facilitate this organism to build up a symbiotic relationship with its insect host, and CRISPR elements that may be used in defense against bacteriophages were discovered.

6.1. Introduction

Acetic acid bacteria (AAB) are best known for their production of acetic acid from ethanol during vinegar or cocoa bean fermentation (Papalexandratou *et al.*, 2013; Wu *et al.*, 2012). Some AAB are also of interest to the industry because of their capacity to produce cellulose or other chemicals such as L-sorbose, involved in the synthesis of vitamin C (Raspor and Goranovič, 2008). Furthermore, AAB occur as plant growth promoting bacteria (Bertalan *et al.*, 2009; Muthukumarasamy *et al.*, 2005), insect endosymbionts (Crotti *et al.*, 2010) or as spoilers of many kinds of beverages such as wine and beer (Wieme *et al.*, 2014b). AAB are classified in the family *Acetobacteraceae* within the *Alphaproteobacteria*. Recent studies of the symbiotic relationship

between AAB and several insect hosts revealed that this symbiosis relies on sugar-based diets such as nectar, fruit sugar or phloem sap (Crotti *et al.*, 2009; Crotti *et al.*, 2010). During a study of bumble bee and honey bee gut microbiota, an *Acetobacteraceae* operational taxonomic unit, referred to as Alpha-2.2, was repeatedly found in the digestive track of honey bees (*Apis* spp.) and bumble bees (*Bombus* spp.). These bacteria were categorized as one of the core bacteria in *B. bimaculatus* (Cariveau *et al.*, 2014; Cox and Gilmore, 2007; Martinson *et al.*, 2011; Mohr and Tebbe, 2006) and its presence in wild bumble bees (*Bombus*) was positively associated with *Crithidia* infection (Cariveau *et al.*, 2014). *Bombella intestini* was recently isolated from the crop of a red-tailed bumble bee, *Bombus lapidarius*, and showed 97–99 % pairwise 16S rRNA gene sequence similarity to Alpha-2.2 (Corby-Harris *et al.*, 2014; Cox and Gilmore, 2007). Simultaneously, a detailed study of Alpha-2.2 bacteria, their source in young larvae of honey bees, and their effect on honey bee larvae fitness was carried out, and included a proposal to name this organism "*Parasaccharibacter apium*" (Corby-Harris *et al.*, 2014). The 16S rRNA sequence of Alpha-2.2 strain A29 (proposed as the type strain of "*P. apium*") showed 98.9 % pairwise sequence similarity with *B. intestini* LMG 28161^{T}, but the former sequence included a 114 nucleotides gap (position 644–757) compared to the latter. The name "*Parasaccharibacter*" was not validated, and given the high level of 16S rRNA sequence similarity, the genus "*Parasaccharibacter*" should be considered a synonym of the genus Bombella (Lapage *et al.*, 1992; Li *et al.*, 2015). The species level relatedness of strain "*P. apium*" A29 and *B. intestini* LMG 28161^{T} requires further taxonomic study. *B. intestini* LMG 28161^{T} showed distinctive phenotypic features from other acetic acid bacteria, such as *Gluconobacter* and *Acetobacter*. In the present study, we examined the genomic characteristics of *B. intestini* LMG 28161^{T} through a whole genome sequencing approach and explored through *in vitro* experiments its capability to oxidize the main components of nectar and honey, *i.e.* sucrose, glucose and fructose (Crotti *et al.*, 2010) and D-mannitol, a six carbon sugar alcohol that is wide distributed in plants (Stoop *et al.*, 1996), under both aerobic and micro-aerobic conditions.

6.2. Materials and methods

Strain cultivation, DNA extraction, genome sequencing, assembly and annotation

Bombella intestini strain LMG 28161^T was cultivated on LMG medium 404 [5 %, w/v, D-glucose; 1 %, w/v, yeast extract (Oxoid) and 1.5 %, w/v, agar] for DNA extraction at large scale using the method of Wilson as modified previously (Cleenwerck *et al.*, 2002). The integrity of the DNA was evaluated on a 1.0 % (w/v) agarose gel and the purity by spectrophotometric measurements at 234, 260 and 280 nm. The DNA concentration was estimated with a Quantus™ fluorometer using a QuantiFluor®ONE ds DNA system kit (Promega Corporation, Medison, USA). Library preparation and genome sequencing were performed by BaseClear BV (Leiden, The Netherlands). Paired-end sequence reads were generated using the Illumina HiSeq2500 system (Illumina Inc., San Diego, CA, USA). The initial *de novo* assembly of the raw reads into contigs was performed using the CLCgenomic workbench v6.5.1 (CLC Inc, Aarhus, Denmark).

Automated gene prediction and annotation of the assembled genome sequences were performed with GenDB v2.2 (Meyer, 2003), the Rapid Annotations using Subsystems Technology (RAST) server (Aziz *et al.*, 2008), and the NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP; `http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html`). The PGAAP gene predictions and annotations were used as basis for the final annotation. They were manually curated for the CDSs of interest using BLASTp (`http://blast.ncbi.nlm.nih.gov/blast`) and UniProt (`http://www.uniprot.org`), taking also into account the information from RAST and GenDB. Metabolic pathways were manually reconstructed using the information from the final annotation. The KEGG database (Kanehisa and Goto, 2000) aided in the reconstruction of the pathways. CRISPRs were searched for using CRISPR Finder (Grissa *et al.*, 2007) and considered if they were classified as "confirmed".

Carbohydrate consumption experiments

To determine the growth rate and glucose consumption rate of strain LMG 28161^T, a 10 L monoculture fermentation experiment was carried out in basal medium [yeast extract 0.5 %, w/v, (Gosselé *et al.*, 1983b)] supplemented with 1 % (w/v) D-glucose. The fermentation was performed in a 15 L BiostatC fermentor (Sartorius AG, Melsungen, Germany) at 28 ℃ and 300 rpm for 36h. Aerobic conditions during the fermentation were ensured by continuously sparging the medium with 5 liters min^{-1} of air. The inoculum for the fermentation experiment was prepared as follows. Strain LMG 28161^T was cultivated in 100 mL LMG medium 404 [5 %, w/v, D-glucose and 1 %, w/v, yeast extract (Oxoid)] and subsequently propagated twice in 400 mL basal medium supplemented with 1 % (w/v) D-glucose to obtain the inoculum. During the inoculum buildup, the transferred volume was always 5 % (v/v) and incubation was done at 28 ℃ for 48 h on a rotary shaker. The inoculum was added to the fermentation vessel aseptically. During the fermentation experiment, the pH was monitored automatically and samples were withdrawn at regular time intervals for offline analysis.

To verify metabolic pathways, the oxidation of the carbohydrates sucrose, D-glucose, D-fructose and D-mannitol under aerobic and micro-aerobic conditions (80 % N_2, 4 % O_2, 8 % H_2 and 8 % CO_2), was verified. The experiments were conducted in triplicate using the same method as described previously for acid production from different carbon sources (Gosselé *et al.*, 1983b) in 50 mL glass bottles filled with 20 mL basal medium supplemented with 1 % (w/v) carbon source, but without bromocresol purple added to the medium. The bottles were incubated at 28 ℃ for 7 days on a rotary shaker, and in a jar for the micro-aerobic conditions experiments. A medium sample was collected before inoculation (without bacterial cells) and after 7 days of incubation with the culture. The samples were centrifuged and the supernatants were stored at -20 ℃until further analyses were carried out.

Analysis of bacterial growth, carbohydrate consumption and metabolite production

Growth of LMG 28161^T during fermentation [(expressed in Log (CFU/mL)) was quantified through plating of 10-fold serial dilutions of the samples in physiological water [0.85 % (w/v) NaCl solution] onto LMG medium M404. Determination of the glucose consumption rate was calculated based on the time of glucose depletion, by measuring glucose concentration every three hours as described previously (Moens *et al.*, 2014). Determination of carbohydrate, acetic acid, D-gluconic acid and 2-keto-D-gluconic acid concentrations in the samples taken from the 50 mL bottles, was done using gas chromatography using a Focus gas chromatograph (Interscience, Breda, The Netherlands) as described previously (Moens *et al.*, 2014).

6.3. Results and discussion

General genome features

The genome sequencing of *B. intestini* strain LMG 28161^T yielded more than 6 million reads of 2 x 100-bp with a genome coverage of 299.0 x. All reads were assembled into 12 contigs of 1,402 to 670,914 nucleotides. Automated gene prediction and annotation of the assembled genome sequences resulted after manual curation in a draft genome of 2.03 Mb with an average G+C content of 54.9 %. The latter value is identical to the DNA G+C content determined through an enzymatic degradation method (Mesbah *et al.*, 1989) and separation of the nucleoside mixture through high performance liquid chromatography (Li *et al.*, 2015). No plasmids were found during the assembly. The final annotation resulted in 1574 coding sequences (CDSs) and 54 RNA sequences, including three rRNA genes (5S, 16S and 23S), 50 tRNA genes and one non-coding RNA. Three clustered regularly interspaced short palindromic repeats (CRISPR) were found on contig 1, contig 2 and contig 5. CRISPR-associated CDSs were found on contig 1 (AL01_08840, AL01_08855) and contig 5 (AL01_03255, AL01_03260, AL01_03265). The draft

genome was submitted to NCBI under the BioProject PRJNA235371.

Metabolic pathways of sugars and sugar derivatives

Based on the annotated draft genome, central metabolic pathways including the Embden-Meyerhof-Parnas (EMP) pathway, the pentose-phosphate pathway (PPP), the pyruvate pathway and tricarboxylic acid (TCA) cycle were reconstructed (Figure 6.1).

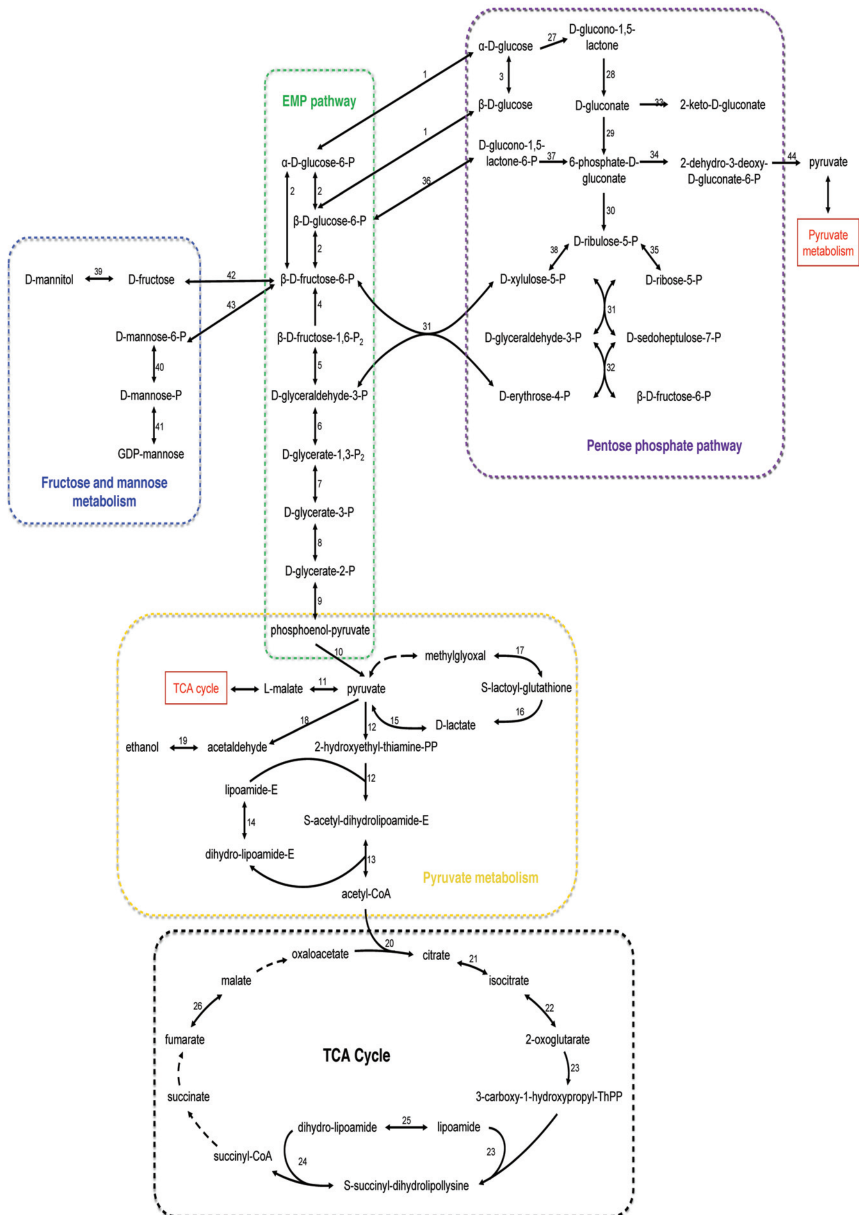
All genes encoding the enzymes of the EMP pathway were identified, except for the phosphofructokinase coding gene, suggesting incomplete glycolysis. The absence of this gene in AAB has been reported before for *Gluconobacter oxydans* 621H, *Acetobacter pasteurianus* IFO 3283 and *Gluconacetobacter diazotrophicus* Pal5^{T} (Azuma *et al.*, 2009; Bertalan *et al.*, 2009; Prust *et al.*, 2005).

All genes encoding the enzymes of the PPP were identified, enabling degradation of hexoses such as glucose and fructose via this pathway. Uptake of hexoses appears possible through a sugar transporter (AL01_-05795 and AL01_06590) or a D-galactose transporter galP (AL01_-03445, AL01_03450 and AL01_02185) which both belong to the major facilitator superfamily (MFS). Phenotypic tests, using the method described previously (Gosselé *et al.*, 1983b), revealed that *B. intestini* LMG 28161^{T} was capable to produce acid from several carbohydrates, including sucrose, D-glucose, D-fructose, D-galactose, D-mannitol and D-mannose (Li *et al.*, 2015). A polyol oxidoreductase (AL01_07080) enabling the conversion of D-mannitol into D-fructose was also found, as well as genes encoding enzymes that catalyze D-mannose utilization (Figure 6.1). This supports the previous observation that *B. intestini* LMG LMG 28161^{T} was able to produce acid from D-mannitol and D-mannose (Li *et al.*, 2015).

D-gluconate can be oxidized to 2-keto-D-gluconate by a membrane-bound gluconate 2-dehydrogenase (AL01_07015) (Figure 6.2). A gene encoding gluconate-5-dehydrogenase was not found. This is in accordance with the previous observation that this organism could produce 2-keto-D-gluconate, but not 5-keto-D-gluconate (Li *et al.*, 2015). A general alcohol dehydrogenase coding gene (AL01_01980) was found,

but not a gene encoding an enzyme for the oxidation of acetaldehyde to acetate. This supports the phenotypic inability of this strain to produce acetate from ethanol (Li *et al.*, 2015). Furthermore, a gene encoding glycerol kinase was not detected, suggesting that glycerol cannot be transferred into the cell and further utilized. This explains why this organism could not grow on, or produce acid from glycerol (Li *et al.*, 2015).

B. intestini LMG 28161^{T} appears to possess an incomplete TCA cycle. Genes coding for enzymes converting succinyl-CoA into succinate, succinate to fumarate and malate to oxaloacetate were not identified. However, L-asparagine permease (AL01_09015) and L-aspartate oxidase (AL01_04960) were identified. The former enables the organism to take up L-asparagine from the environment, which can then be hydrolyzed to L-aspartate. L-aspartate oxidase is a flavoprotein (FAD) that acts on the CH-NH_2 group of donors with O_2 as electron accepter. O_2 can be replaced by fumarate as electron acceptor, yielding succinate (Bossi *et al.*, 2002). The ability of the enzyme to use both O_2 and fumarate in cofactor reoxidation enables it to function under both aerobic and anaerobic conditions (Bossi *et al.*, 2002). L-aspartate can be converted by aspartate aminotransferase (AL01_03035) to oxaloacetate to join the TCA cycle. As for fumarate, it can also be converted from L-aspartate via two different 2-step reactions with adenylosuccinate or L-argininosuccinate as intermediates, catalyzed by adenylosuccinate synthetase (AL01_06765), adenylosuccinate lyase (AL01_00960), argininosuccinate synthase (AL01_09265) and argininosuccinate lyase (AL01_02240). Although the three above mentioned substrates of the TCA cycle can be generated by other reactions, the amount of energy generated through the TCA cycle may be rather limited as in a complete TCA cycle, the three enzymatic reactions catalyzed by these three missing enzymes are accompanied by the generation of GTP, $FADH_2$ or NADH. The absence of a complete TCA cycle and the inability to oxidize acetic acid to CO_2 and water corresponds with other AAB, such as *Gluconobacter* species which also have a nonfunctional TCA cycle (Prust *et al.*, 2005; Raspor and Goranovič, 2008).



EMP pathway
α-D-glucose-6-P
β-D-glucose-6-P
β-D-fructose-6-P
β-D-fructose-1,6-P2
D-glyceraldehyde-3-P
D-glycerate-1,3-P2
D-glycerate-3-P
D-glycerate-2-P
phosphoenol-pyruvate
Fructose and mannose metabolism
D-mannitol
D-fructose
D-mannose-6-P
D-mannose-P
GDP-mannose
Pentose phosphate pathway
α-D-glucose
β-D-glucose
D-glucono-1,5-lactone
D-gluconate
2-keto-D-gluconate
D-glucono-1,5-lactone-6-P
6-phosphate-D-gluconate
2-dehydro-3-deoxy-D-gluconate-6-P
pyruvate
Pyruvate metabolism
D-ribulose-5-P
D-xylulose-5-P
D-ribose-5-P
D-glyceraldehyde-3-P
D-sedoheptulose-7-P
D-erythrose-4-P
β-D-fructose-6-P
Pyruvate metabolism
TCA cycle
L-malate
pyruvate
methylglyoxal
S-lactoyl-glutathione
D-lactate
ethanol
acetaldehyde
2-hydroxyethyl-thiamine-PP
lipoamide-E
S-acetyl-dihydrolipoamide-E
dihydro-lipoamide-E
acetyl-CoA
TCA Cycle
oxaloacetate
citrate
isocitrate
malate
2-oxoglutarate
fumarate
3-carboxy-1-hydroxypropyl-ThPP
succinate
dihydro-lipoamide
lipoamide
succinyl-CoA
S-succinyl-dihydrolipollysine

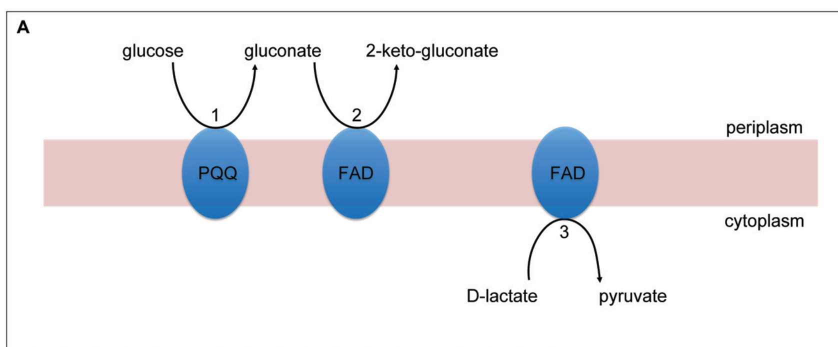
Figure 6.1. Central metabolic pathways of *Bombella intestini* LMG 28161^T. 1, glucokinase(AL01_01675); 2, glucose-6-phosphate isomerase(AL01_06115); 3, aldoseepimerase(AL01_04275); 4, fructose 1, 6-bisphosphatase (AL01_-08695); 5, fructose-bisphosphatealdolase(AL01_06890); 6. glyceraldehyde-3-phosphate dehydrogenase (AL01_03750); 7. phosphoglyceratekinase (AL01_-03755); 8, phosphoglyceratemutase(AL01_07435, AL01_07655); 9, enolase(AL01_00860); 10, pyruvatekinase (AL01_00625); 11, malatedehydrogenase (AL01_05845); 12, pyruvatedehydrogenase (AL01_00915, AL01_-00920, AL01_03860); 13, pyruvatedehydrogenase E2 (AL01_00925)14, dihydrolipoamidedehydrogenase (AL01_00930); 15, lactatedehydrogenase (AL01_-06935); 16, hydroxyacylglutathionehydrolase(AL01_04950); 17, lactoylgluthathionelyase(AL01_00090); 18, pyruvatedecarboxylase(AL01_08375); 19, alcohol dehydrogenase (AL01_01980, AL01_07015); 20, citratesynthase(AL01_06255); 21, aconitatehydratase1 (AL01_06260); 22, $NADP^+$-dependentisocitratedehydrogenase (AL01_06250); 23, 2-oxoglutarate dehydrogenase E1 (AL01_08340); 24, dihydrolipoyllysinesuccinyltransferase (AL01_07740); 25, dihydrolipoamidedehydrogenase (AL01_00930); 26, fumaratehydratase(AL01_05840); 27, PQQ-dependentglucose dehydrogenase (AL01_09305); 28, gluconolactonase(AL01_06230); 29, lactatedehydrogenase (AL01_06935); 30, phosphogluconatedehydrogenase (AL01_-06120); 31, transketolase(AL01_06110); 32, transaldolase(AL01_06115); 33, gluconate2-dehydrogenase (AL01_07015); 34, 6-phosphogluconate dehydrogenase (AL01_06120); 35, ribose-5-phosphate isomerase(AL01_-06135); 36, glucose-6-phosphate dehydrogenase (AL01_02790); 37, 6-phosphogluconolactonase (AL01_06130); 38, ribulose-phosphate3-epimerase (AL01_09060); 39, polyol:NADPoxidoreductase(AL01_07080); 40, phosphomannomutase(AL_0102400); 41, mannose-1-phosphate guanyltransferase(AL01_07360); 42, carbohydratekinase (AL01_03675); 43, mannose06-phosphate isomerase(AL01_00140); 44, 2-dehydro-3-deoxyphosphogluconate aldolase(AL01_04330).

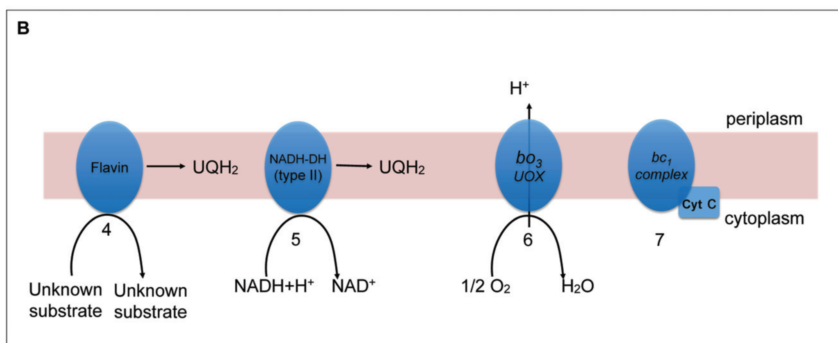
Membrane-bound dehydrogenase and respiratory chain

Compared to other genome sequenced AAB strains, *B. intestini* LMG 28161^T does not seem to possess many membrane-bound dehydrogenases, as only three were found (Figure 6.2A), namely a cofactor pyrroloquinoline quinone (PQQ) dependent glucose dehydrogenase (AL01_09305)

allowing the conversion of glucose into gluconate, a flavine adenine dinucleotide (FAD) dependant gluconate 2-dehydrogenase (AL01_07015) allowing the conversion of gluconate into 2-keto-gluconate, and a FAD dependant D-lactate oxidase (AL01_06935) allowing the conversion of D-lactate into pyruvate. A *pqqBCDE* operon (AL01_07315, AL01_07320, AL01_07325 and AL01_07330) encoding proteins for the synthesis of the cofactor PQQ was detected. In the genomes of *G. oxydans* 621H and *A. pasteurianus* 386B a *pqqABCDE* operon is present. It was shown previously, that a *pqqA* mutant of *G. oxydans* 621H was unable to grow on D-mannitol, D-glucose or glycerol as the sole energy source (Chouaia *et al.*, 2014). *B. intestini* LMG 28161^T can grow on D-glucose and D-mannitol as the sole energy source (see below), which indicates that *pqqA* is not vital for this strain.
Genes encoding ubiquinol-cytochrome c reductase (*bc1* complex) (AL01_05885, AL01_08145, AL01_08150) and cytochrome c (AL01_-05875) were found in the genome, but not cytochrome c oxidase encoding genes. Genes encoding a type II NADH dehydrogenase (AL01_-05990) and a NAD(P)H:ubiquinone oxidoreductase (AL01_07780) were present in the genome. Both these enzymes catalyze electron transfer from NADH to ubiquinone. A flavoprotein-ubiquinone oxidoreductase (AL01_08300) catalyzes electron transfer from flavoprotein to ubiquinone. The reduced product, ubiquinol, can diffuse within the membrane and be re-oxidized by cytochrome *bo3* ubiquinole oxidase (AL01_00470, AL01_00475, AL01_00480 and AL01_00485) (Figure 6.2B). A previous study suggested that AAB acquired ubiquinol oxidase from β/γ-Proteobacteria via horizontal gene transfer and created afterwards a truncated respiratory chain in which electron transfer to oxygen occurs via ubiquinol oxidase directly, accepting electrons from ubiquinol. The truncated respiratory chain would generate less energy, but allows rapid oxidations, which would be beneficial for AAB (Matsutani *et al.*, 2014). Cytochrome *bo3* oxidase was detected in other AAB genomes and shows a high affinity for oxygen, possibly allowing their survival in environments with low oxygen availability, such as the insect gut (Chouaia *et al.*, 2014).

Figure 6.2. A) Membrane bound dehydrogenase and B) respiratory chain of *Bombella intestini* LMG 28161^T.
1, membrane-boundglucose dehydrogenase (AL01_09305); 2, gluconate2-dehydrogenase (AL01_07015); 3, membrane-boundlactatedehydrogenase (AL01_06935); 4, electrontransfer flavoprotein-ubiquinoneoxidoreductase(AL01_08300); 5, type II NADH dehydrogenase (AL01_05990); 6, cytochrome *bo3* ubiquinoloxidase (AL01_00470, AL01_-00475, AL01_00480 andAL01_00485); 7, ubiquinol-cytochromec reductase (*bc1* complex) (AL01_05885, AL01_08145, AL01_08150); 8, CytC, cytochrome c (AL01_05875).





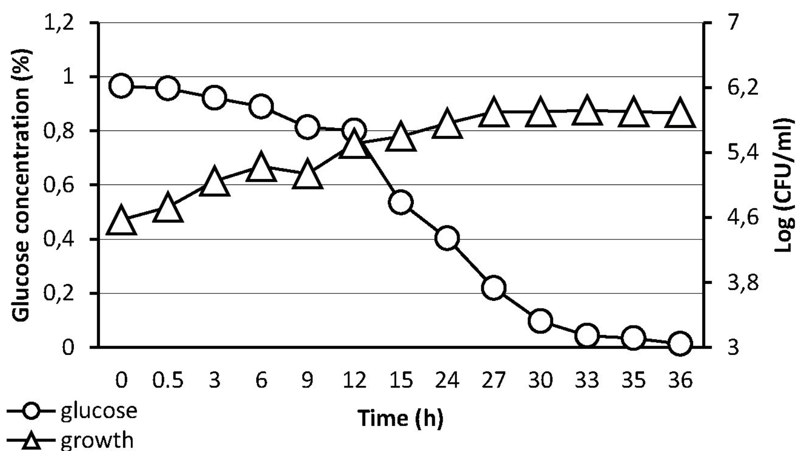
Bumble bee endosymbionts related features

ABC transporters and type IV secretion systems have been reported to be involved in the cross talk between endosymbionts and their insects host (Backert *et al.*, 2015; Chouaia *et al.*, 2014; Kwong *et al.*, 2014; Low *et al.*, 2014). Multiple CDSs associated to ABC transporters were identified (Table 1). Type IV secretion system protein coding regions (AL01_03185, AL01_03190, AL01_03195, AL01_03200, AL01_03220, AL01_03225 and AL01_03230) were also detected in the genome. A signal recognition partical (SRP) complex (AL01_03075 and AL01_-06750), which recognizes and targets specific proteins on the plasma membrane, was also present. The restriction modification system to degrade foreign DNA, that was found in the genomes of two additional *Bombus* endosymbionts, *Gilliamella apicola* and *Snodgrasella alvi* (Kwong *et al.*, 2014), was not detected in the genome of *B. intestini* LMG 28161^T; yet CRISPR elements were present and may be used in defense against bacteriophages.

Metabolite analysis

A ten liter monoculture fermentation experiment was carried out in basal medium [yeast extract 0.5 %, w/v, (Gosselé *et al.*, 1983b)] supplemented with 1 % (w/v) D-glucose to determine the growth rate and D-glucose consumption rate of strain LMG 28161^T. During this experiment, *B. intestini* LMG 28161^T oxidized D-glucose from the beginning of the fermentation, with a rapid drop after 12 hours until D-glucose was completely depleted after 36h (Figure 6.3). The biomass accumulation during fermentation was limited [a log (CFU/mL) increase from 4.5 to 5.9], which was probably due to the composition of medium that was nutritionally limited. In addition, the pH value of the medium decreased from 7.0 to 3.5 during the fermentation, which supported the previous observation that this organism was capable to produce acid from D-glucose (Li *et al.*, 2015).
Bumble bees feed on nectar, which comprises sucrose, fructose, glucose and in some plants also D-mannitol (Lohaus and Schwerdtfeger,

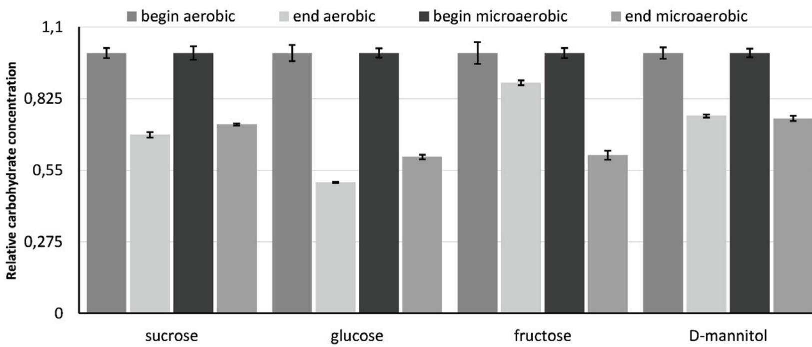
Figure 6.3. Growth and D-glucose consumption by *Bombella intestini* LMG 28161^T during fermentation in basal medium with 1 % (w/v) D-glucose.



2014) as main components; the latter two carbohydrates are also often used as main carbon source in AAB growth media. Therefore, these carbohydrates may serve as carbon source for *B. intestini* during its endosymbiotic lifestyle. As the digestive track of bees is a micro-aerobic environment we aimed to examine growth on sucrose, D-glucose, D-fructose and D-mannitol in both aerobic and micro-aerobic conditions. To this end 20 mL of basal medium supplemented with 1 % of each carbohydrate was inoculated with *B. intestini* LMG 28161^T and the amount of carbohydrate was determined before and after 7 days of incubation with the strain under both aerobic and micro-aerobic conditions. *B. intestini* LMG 28161^T was capable to utilize these four carbohydrates, however, the amount of sucrose and D-glucose was slightly more decreased under aerobic than under micro-aerobic conditions, whereas D-fructose seemed to be utilized slightly more under micro-aerobic conditions (Figure 6.4). In addition, 2-keto-D-gluconic acid was detected when sucrose and D-glucose were used as the sole carbon source. Gluconic acid and acetic acid were not detected. The *in vitro* experiments

therefore confirmed that *B. intestini* LMG 28161^T was able to produce 2-keto-D-gluconic acid and oxidize these substrates under both aerobic and micro-aerobic conditions.

Figure 6.4. Carbohydrate consumption by *Bombella intestini* LMG 28161^T under aerobic and micro-aerobic conditions.



In the 10 L fermentation experiment, 1 % D-glucose was depleted completely after 36 hours of incubation, whereas none of the 4 carbohydrates was depleted in the 20 mL experiments which were executed in 50 mL glass bottles. During cultivation in the 10 L fermenter, aerobic conditions were ensured by continuously sparging the medium with 5 liters min^{-1} of air, which was not the case for the bottle tests where growth media were stirred on a rotary shaker and growth mainly occurred at the surface. This difference in aeration might explain the difference in carbohydrate depletion in the two test systems. The lack of effective aeration in small scale carbohydrate consumption experiments may explain the lack of reproducibility of such results reported in taxonomic studies of AAB (Cleenwerck and De Vos, 2008; Li *et al.*, 2015; Spitaels *et al.*, 2014a).

6.4. Conclusion

The genome size of *B. intestini* LMG 28161^T, an endosymbiotic acetic acid bacterium in bumble bees, is 2.03 Mb, which is small compared to those of other AAB genomes that have been determined (2.7 - 3.9 Mb) (Azuma *et al.*, 2009; Greenberg *et al.*, 2007; Illeghems *et al.*, 2013a; Kubiak *et al.*, 2014; Prust *et al.*, 2005). A small genome size is a typical feature of bacterial endosymbionts, and may suggest that *B. intestini* has well adapted to the bumble bee digestive track (Corby-Harris *et al.*, 2014; Kwong *et al.*, 2014; Nilsson *et al.*, 2005). The reconstructed metabolic pathways were congruent with its phenotypic features determined previously (Li *et al.*, 2015) and in the frame of the present study: the strain is capable of oxidizing sucrose, D-glucose, D-fructose and D-mannitol, which are all present in nectar or honey, while it is incapable of oxidizing ethanol or glycerol, which are also not available in bumble bee gut. In addition, the metabolite experiment carried out revealed the oxidation capacity of the strain towards different carbohydrates under aerobic condition differed from that of under micro-aerobic condition.

6.5. Acknowledgements

The BCCM/LMG collection is supported by the Federal Public Planning Service-Science Policy, Belgium. The authors acknowledge the financial support of the Research Foundation-Flanders (FWO-Vlaanderen), the Research Council of Ghent University and the Vrije Universiteit Brussel (SRP, IRP, and IOF projects), and of the Hercules Foundation. L.L. acknowledges the Chinese Scholarship Council and Ghent University Co-Funding.

Part IV.

General Discussion and Future Perspectives

Preamble

The main goals of this work were to evaluate MALDI-TOF MS as a fast, accurate and cost-effective tool for identification and classification of acetic acid bacteria (AAB), assess the value of whole genome sequence analysis for AAB taxonomy and explore alternative isolation strategies for AAB. This part presents a general discussion of the results obtained in the past four years and provides future perspectives.
In **Chapter 7**, the effectiveness of MALDI-TOF MS as a fast and accurate method for identification of AAB was evaluated and its usefulness to reveal novel AAB species and differentiate non AAB was assessed. In addition, the potential of MALDI-TOF MS for AAB taxonomy were discussed.
In **Chapter 8**, the value of the current polyphasic taxonomy approach applied in AAB taxonomy, and used for the characterization of several AAB species (**Chapters 3, 4** and **5**), was assessed. This includes an evaluation of the usefulness of MLSA, which was applied in **Chapter 4** to the genus *Acetobacter* and was in fact an extension of the existing MLSA scheme. Furthermore, the value of whole genome sequence analysis (used in **Chapters 3** and **6**) for AAB taxonomy was evaluated. The possibilities to incorporate whole genome sequences into the description of novel AAB species were discussed.

7 MALDI-TOF MS: a rapid, high-throughput approach for classification and identification of AAB

7.1. Call for a quick identification method for acetic acid bacteria

One of the main aims of the present study was to develop and evaluate a robust MALDI-TOF MS database for identification and classification of novel AAB isolates. AAB are widespread in nature and often involved in food and beverage processes, where they play a beneficial or detrimental role. Some of them are opportunistic human pathogens, while others (especially *Gluconobacter* strains) are interesting for the industry because of their oxidizing capacity. Their species identity is crucial for clinical and biotechnological process control, to decide on the appropriate actions to undertake (Trček and Barja, 2014). However, the

current identification methods are mostly DNA-based and often time-consuming. Particularly, when dealing with a large number of isolates, those DNA-based methods clearly fail to identify isolates sufficiently quickly. As an additional consequence, the workload and cost will be overwhelming.

7.2. MALDI-TOF MS for AAB identification and classification

Since the suggestion to apply mass spectrometry in bacterial identification in the 1970s, there has been an increase of MALDI-TOF MS applications for identification and classification of *Bacteria*, *Archaea*, yeasts and fungi (Claydon *et al.*, 1996; Holland *et al.*, 1996; Krishnamurthy and Ross, 1996; Schumann and Maier, 2014). With respect to AAB, MALDI-TOF MS has been evaluated for the quick identification of vinegar fermentation and beer spoilage related AAB (Andrés-Barrao *et al.*, 2013; Wieme *et al.*, 2014b), although these studies addressed only a limited number of species and strains. In the present study, we generically evaluated the usefulness of MALDI-TOF MS for AAB identification and classification. First, a robust AAB identification database was constructed using a large set of well-identified AAB reference strains. Subsequently, the established database was validated for identification using AAB from various sources, including novel AAB isolates and more than 200 poorly characterized AAB strains present in the BCCM/LMG Bacteria Collection (`http://bccm.belspo.be`). Finally, we used the database as a dereplication and identification tool in our study on various isolation procedures for these bacteria.

7.2.1. Instrument and software requirements,cultivation conditions, sample preparation

The accuracy of bacterial identification using MALDI-TOF MS highly depends on the robustness of the database used and the available software tools to compare the mass spectra. The commercially available

MALDI Biotyper (Bruker Daltonics), VITEK MS (bioMerieux) and Andromas (Andromas SAS) systems all contain integrated software packages with a proprietary database of mainly clinically relevant microorganisms. In our research, a 4800 Plus MALDI TOF/TOM™ Analyzer (AB Sciex, Framingham, MA, USA) was used for the generation of mass spectra. This instrument was not designed for bacterial identification purposes and contains therefore no identification database or data analysis software.

The reference AAB database constructed in the present Ph.D study comprises all presently known species of the family *Acetobacteraceae* (June 2015) except for two recently reported species, *i.e. Swingsia samuiensis* and *Nguyenibacter vanlangensis*. Moreover, as culture conditions can affect the mass spectra (Giebel *et al.*, 2010; Schumann and Maier, 2014), reference strains were as much as possible cultivated in standardized conditions. Two media (LMG medium 13 and LMG medium 404) were found appropriate to cultivate most reference strains. To cover for variations caused by growth media, spectra from both LMG medium 13 and LMG medium 404 were generated for each reference strain, when enough growth could be obtained.

The mass spectra were imported into the BioNumerics software package (Applied Maths, Sint-Martens-Latem, Belgium) for further analyses. In the beginning of the database construction, the software package BioNumerics version 5.1 was used, which primarily allowed curved-based analysis by means of the Pearson product-moment correlation coefficient and the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster algorithm. Although most of the reference AAB strains clustered according to their species designation, several sets of closely related species could not be differentiated, and species with an established genetic heterogeneity tended to split into multiple clusters. The obtained clustering was furthermore poorly representative of the organisms' phylogenetic relationships as revealed through comparative sequence analysis of conserved chronometers such as the 16S rRNA gene. Later on, with the upgrade of the BioNumerics software package to version 7.1, a peak-based algorithm could be used, which matches all peaks in the mass spectra to a peak class using constant and

linearly varying tolerance values, respectively. The obtained dataset was converted into a binary character set and analyzed using the Dice coefficient and clustered using UPGMA. In contrast to the curve-based analysis, the peak-based algorithm resulted in a much clearer species and genus level differentiation; moreover, the obtained dendrogram also reflected the phylogeny within the family *Acetobacteraceae* to a certain extent (**Chapter 3**). This is not entirely unexpected as MALDI-TOF MS profiles are dominated by ribosomal proteins, of which the structure is conservative enough to infer the phylogenetic relationship of bacteria (Schumann and Maier, 2014).
Sample preparation is also a crucial step in MALDI-TOF MS, as it determines the number of peaks obtained, the signal-to-noise (S/N) ratio and the discriminatory power of the spectra. In addition, it affects the reproducibility, reliability, turn-around time and cost of the analysis. Generally speaking, there are two strategies for sample preparation, the intact cells method and the protein extraction method. The former is widely used in applications of MALDI-TOF MS for bacterial identification as it is straightforward and requires very short handling time. However, comparisons of different sample preparation techniques revealed that the extraction of proteins prior to the measurement resulted in an increased identification accuracy in comparison with the intact cell method (Fournier *et al.*, 2012; Schulthess *et al.*, 2013). In addition, a lot of AAB strains produce polysaccharides (including cellulose) during growth, which can reduce the profile quality dramatically. Taking this into account, we decided to use the protein extraction method in the present study. In practise, the majority of AAB strains analyzed yielded MS profiles of good quality (signal intensity > 500, peak number >5 and slope < 30).

7.2.2. Taxonomic resolution of MALDI-TOF MS

Despite the increasing application of MALDI-TOF MS in microbiology, part of its taxonomic resolution is still under debate. One way to clarify this is to compare the taxonomic resolution of MALDI-TOF MS with that of established tools for classification, identification and typing

of bacteria (Schumann and Maier, 2014). Previous studies revealed that MALDI-TOF MS has a limited capability to reveal phylogenetic relationships of distantly related organisms, *i.e.* genera or higher taxonomic ranks. The main application of MALDI-TOF MS lies in the differentiation and identification of species. For subspecies or strain level identification, more sophisticated analyses are required (Busse *et al.*, 2012; De Bruyne *et al.*, 2011; Dieckmann *et al.*, 2008; Ruiz-Moyano *et al.*, 2012; Schumann and Pukall, 2013; Tanigawa *et al.*, 2010; Welker and Moore, 2011).
As mentioned above, in the present study, numerical analysis of the obtained reference spectra reflected the phylogeny of the family to a certain degree. For instance, the closer phylogenetic relationship of *Gluconacetobacter* and *Acetobacter*, compared to *Gluconobacter*, was reflected in the MALDI-TOF MS based cluster analysis (Figure 3.1, **Chapter 3**). As expected, most of the strains grouped in a species specific cluster, confirming thereby the anticipated paradigm regarding the taxonomic resolution of MALDI-TOF MS. It is however noteworthy that this did not apply equally to all analyzed organisms as, for instance, several *Asaia* species clustered together. This supports the previous assumption that the taxonomic resolution of MALDI-TOF MS profiling is taxon-dependent (Ghyselinck *et al.*, 2011; Sandrin *et al.*, 2013). Further it was also noticed that the algorithms used to perform the comparisons also had impact on the taxonomic resolution, as for instance three *Asaia* species could be differentiated using the curve-based Pearson correlation coefficient, while only two species could not be differentiated when using the peak-based method, this way resulting in a taxonomically clearer clustering.
Although this was not specifically addressed in the studies presented in **Chapters 3, 4** and **5**, our MALDI-TOF MS data revealed the potential to differentiate below the species level. For example, mass spectra of strains belonging to *Gluconacetobacter sacchari*, *Gluconacetobacter diazotrophicus*, *Komagataeibacter europaeus* or *Komagataeibacter saccharivorans* not only grouped at the species level, but also on the strain level (Figure 7.1). In the case of *Ga. sacchari*, *Ga. diazotrophicus* and *Kom. saccharivorans* this was only observed when the profiles originated

from cells cultivated on the same medium, whereas for *Kom. europaeus* the profiles grouped per strain regardless of the growth medium (Figure 7.1). These results support the previous statement that the influences of the growth medium on the profiles have no real impact on species level differentiation, but mainly affect the potential for strain level differentiation (Wieme *et al.*, 2014a).

In order to obtain strain level identification, the presence and recognition of strain specific peaks in mass spectra are crucial. Overall, strains of the same species share a large fraction of the peaks present in the mass spectra. Yet, strain specific peaks are generally present. However, such strain specific peaks often appear to be minor peaks with a relatively low signal intensity (Figure 7.2, **Chapter 3** Figure S3.1), and the currently used algorithms (Pearson correlation coefficient and Dice coefficient) do not give (enough) weight to such small peaks to enable strain differentiation. Differentiation below the species level may be further improved, for example by screening for strain-specific peaks as biomarkers or by enhancing the weight of biomarker peaks in comparison to non-specific signals (Schumann and Maier, 2014).

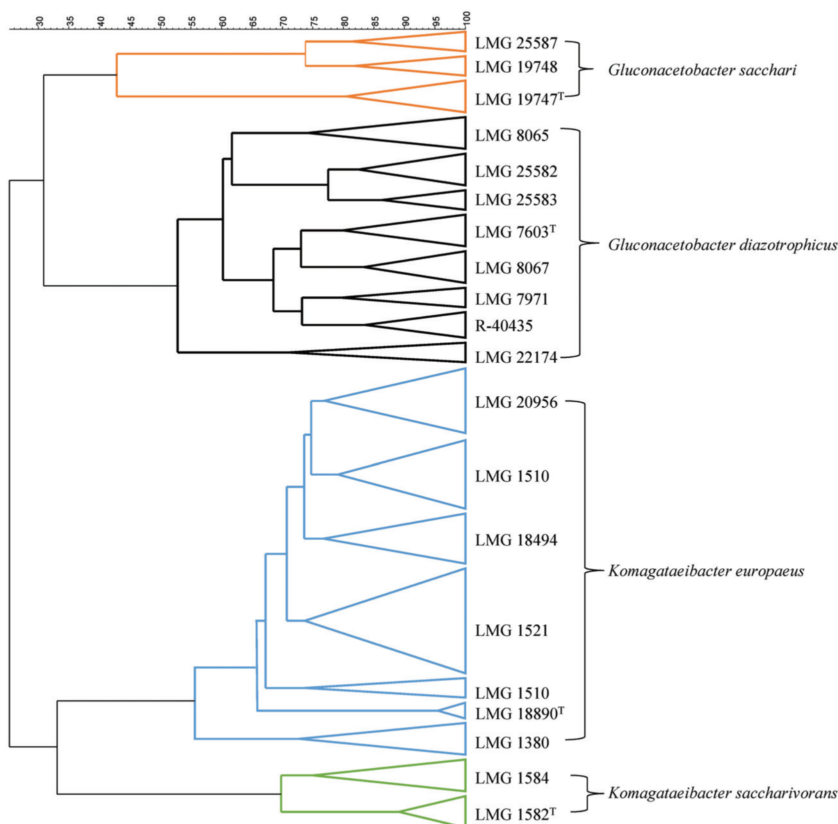


Figure 7.1. Dendrogram based on MALDI-TOF MS profiles of strains belonging to *Gluconacetobacter sacchari*, *Gluconacetobacter diazotrophicus*, *Komagataeibacter europaeus* or *Komagataeibacter saccharivorans*. Profiles of *Ga. sacchari*, *Ga. diazotrophicus* and *Kom. saccharivorans* originated from cells cultivated on LMG medium 13. Profiles of *Kom. europaeus* were originated from cells cultivated from multiple media (LMG medium 13, LMG medium 404 and LMG medium 405). Profile similarities were calculated by the peak-based Dice coefficient.

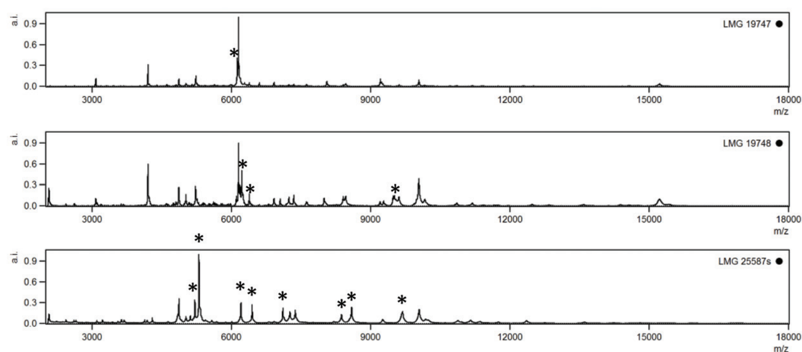


Figure 7.2. MALDI-TOF MS profiles of *Gluconacetobacter sacchari* strains. *, strain specific peaks present in each strain.

7.3. MALDI-TOF MS allows to discover novel AAB

MALDI-TOF MS is increasingly used as a novel tool in polyphasic taxonomy (Vandamme and Peeters, 2014). For instance, from the year 2010 on, the description of almost 80 novel taxa in the International Journal of Systematic and Evolutionary Microbiology included the use of MALDI-TOF MS data as discriminatory characteristics. In the frame of the present study, isolates from cocoa bean fermentation, kefir and the bumble bee gut, but also a large collection of poorly characterized strains from the BCCM/LMG Bacteria Collection were subjected to MALDI-TOF MS and their profiles were compared to those in the constructed MALDI-TOF MS AAB database. Isolates or strains with profiles that fell into a cluster containing reference strains of the same species and that showed a high similarity to those AAB reference strains after numerical analysis, were considered identified. Isolates or strains that clustered separately but among the reference species, were hypothesized to represent novel AAB species and were further examined by 16S rRNA or housekeeping gene sequencing. Two of these potentially novel AAB species were fully characterized and described as *Acetobacter sicerae* sp. nov. and *Bombella intestini* gen. nov., sp. nov. Their descriptions indeed included MALDI-TOF MS profiles of the new species and of their phylogenetic neighbor species (**Chapter 4** and **5**). A considerable fraction of our isolates was identified as belonging to very recently described species such as *Acetobacter persici*, *Acetobacter okinawensis*, *Acetobacter sicerae* and *Gluconacetobacter takamatsuzukensis*, which were first reported between 2012 and 2014 (Iino *et al.*, 2012a; Li *et al.*, 2014; Nishijima *et al.*, 2013).
In contrast, isolates that formed clusters well separated from those of the established AAB species were consistently non-AAB, as confirmed by 16S rRNA gene sequencing (**Chapter 3**).

Additional novel AAB species discovered by MALDI-TOF MS in the present study

In the study presented in **Chapter 3**, nine additional strains from the BCCM/LMG Bacteria Collection formed six separate clusters among the reference AAB species. Based on their nearest neighbors in the MALDI-TOF MS database, they are tentatively considered as novel species in the genera *Acetobacter*, *Asaia*, *Bombella* and *Gluconobacter*, respectively (Table 7.1). Their assignment to these genera was confirmed by subsequent 16S rRNA or housekeeping gene sequence analysis (**Chapter 3**), but they were not further characterized in the frame of the present Ph.D study.
Two of these strains, *i.e.* LMG 1352 and LMG 1354, were isolated from a beehive and clustered close to *Bombella intestini*, which commonly occurs in honey bees and bumble bees. Partial 16S rRNA gene sequence analysis of strain LMG 1354 revealed its close relationship (97-100 % sequence similarity by blast search) with Alpha-2.2 bacteria and with *Bombella intestini* (98 % sequence similarity) (Figure 7.3).
Five strains represent three putative novel *Acetobacter* species, one of which was isolated from beer or brewery samples in Belgium and the U.K. (the biological origin of strains of the remaining two species is unknown). They grouped with *A. aceti*, *A. oeni* and *A. sicerae*, as nearest phylogenetic neighbor species as determined through MLSA experiments. Finally, one isolate from a rotting apple as identified as a putative novel *Asaia* species with *As. siamensis* as nearest neighbor species, and a last isolate of unknown origin was recognized as a putative novel *Gluconobacter* species with *G. cerevisiae* as nearest neighbor species (Table 7.1)

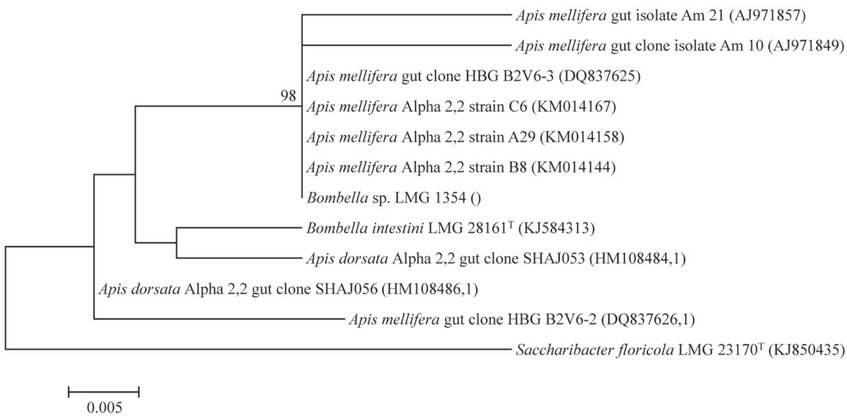


Figure 7.3. Maximum-likelihood tree based on nearly complete [partial for LMG 1354 (381 nt)] 16S rRNA gene sequences (1396 nt), showing the phylogenetic relationships of strains within the genus *Bombella*. The substitution model used was the Kimura 2-parameter model. The robustness of the branching is indicated by bootstrap values calculated for 1000 subsets. Bar, 0.5 % sequence divergence.

Table 7.1. List of novel AAB discovered by MALDI-TOF MS and confirmed by 16S rRNA or housekeeping gene sequence analysis.

Strain number	Species name according to catalogue	Classified as	Accession number of available sequenc				Geographic origin	Biological origin
			dnaK	***groEL***	***rpoB***	**16S rRNA gene**		
1627	*Acetobacter pasteurianus*	*Acetobacter sp.* nov. I	KR677146	KR677137	KR677149			
1636	*Acetobacter* sp.	*Acetobacter* sp. nov. II	KR677147	KR677138	KR677150			Starch producing mutant of an *Acetobacter pasteurianus* strain
1637	Unidentified	*Acetobacter* sp. nov. II ¶						
1506	*Acetobacter aceti*	*Acetobacter* sp. nov. III	KR677141	KR677135			Belgium	Beer
1706	*Acetobacter* sp.	*Acetobacter* sp. nov. III ¶					United Kingdom	Brewery
1453	*Gluconobacter oxydans*	*Asaia* sp. nov.	KR677140	KR677134				
1352	*Acetobacter* sp.	*Bombella* sp. nov. ¶					Madrid area, Spain	Beehive
1354	*Acetobacter* sp.	*Bombella* sp. nov.				x	Madrid area, Spain	Beehive
1745	*Gluconobacter oxydans*	*Gluconobacter* sp. nov.				x	Ghent, Belgium	Rotting apple

Strain number	Species name according to catalogue	Classified as	Closest GenBank database hit and nucleotide identity (%)				Nearest neighbor in MALDI-TOF MS cluster
			dnaK	***groEL***	***rpoB***	**16S rRNA gene**	
1627	*Acetobacter pasteurianus*	*Acetobacter sp.* nov. I	*A. aceti*, 93 %	*A. aceti*, 93 %	*A. aceti*, 83 %		*A. aceti*
1636	*Acetobacter* sp.	*Acetobacter* sp. nov. II	*A. oeni*, 90 %	*A. oeni*, 93 %	*A. estunensis*, 83 %		*A. oeni*
1637	Unidentified	*Acetobacter* sp. nov. II ¶					*A. oeni*
1506	*Acetobacter aceti*	*Acetobacter* sp. nov. III	*A. sicerae*, 96 %	*A. sicerae*, 98 %			*A. sicerae*
1706	*Acetobacter* sp.	*Acetobacter* sp. nov. III ¶					*A. sicerae*
1453	*Gluconobacter oxydans*	*Asaia* sp. nov.	*As. siamensis*, 93 %	*As. siamensis*, 92 %			*As. astilbes*
1352	*Acetobacter* sp.	*Bombella* sp. nov. ¶					*B. intestini*
1354	*Acetobacter* sp.	*Bombella* sp. nov.				*Acetobacteraceae* DAT819, 100%	*B. intestini*
1745	*Gluconobacter oxydans*	*Gluconobacter* sp. nov.				*G. cerevisiae* 99%	*G. kondonii*

¶ Identified by MALDI after identification of strains of the same MALDI cluster through housekeeping gene sequence analysis.

Despite the advantage of recognizing potentially novel taxa using MALDI-TOF MS, no formal criteria or threshold levels for the delineation of species and genera by MALDI-TOF MS have been established. The commercially available MALDI Biotyper software provides an identification score when analyzing novel isolates. However, the species delineation threshold varies according to different bacterial groups, not to mention that the software and database do not allow to recognize organisms that are not present in the reference database, so there is no generic implementation of such scores in systematics (Schumann and Maier, 2014).

7.4. Isolation strategies for AAB and MALDI-TOF MS for the dereplication of AAB isolates

In the frame of the present study, we investigated direct isolation and enrichment approach for AAB isolation using bumble bee guts as sources(**Chapters 5**). Both acidified and non-acidified media, with varying carbon sources (such as LMG medium 13, LMG medium 404, LMG medium 405 and mDMS), were used as the former encourages the growth of *Acetobacter*, *Gluconacetobacter* species, while the latter allows the growth of species unable to grow in the presence of acetic acid, such as *Asaia* sp. Direct isolation and enrichment approaches were also compared, where in the former, samples were diluted and plated directly on the selective media, while in the latter approach microorganisms were allowed to grow in a liquid enrichment medium prior to plating on the selective media. In our experiments, "*Commensalibacter*" were picked up by direct isolation on two non-acidified agar media LMG medium 13 and LMG medium 404, while *Asaia astilbes* and a novel AAB *Bombella intestini* gen. nov., sp. nov. was only picked up from one out of four applied selective media after aerobic incubation at 28 ℃ after enrichment in alcohol free, non-acetified enrichment medium. The latter microorganism was also capable to grow under micro-aerobic conditions and at 37 ℃. Hence, the use of a variety of selective media, micro-

aerobic cultivation conditions and an increased incubation temperature may lead to the discovery of more novel AAB species in the future as has already been proven successful by Corby-Harris and co-workers (Corby-Harris *et al.*, 2014).
Furthermore, in the course of isolating AAB from bumble bee guts, lactic acid bacteria were initially found besides AAB on the AAB selective agar media. To inhibit their growth, sodium deoxycholate was added to all selective agar media afterwards. Sodium deoxycholate is a bile salt to which the cell walls of most Gram-positive bacteria are susceptible, while the cell walls of Gram-negative bacteria are resistant. To estimate which concentration to use, AAB and LAB were cultivated on agar media supplemented with sodium deoxycholate of different concentrations. The results showed that 0.02 % sodium deoxycholate was effectively inhibiting the growth of LAB, while it showed only minor effect on the growth of AAB.
Whether or not AAB are 100 % selectively isolated, MALDI-TOF MS allows a rapid grouping of isolates into several clusters. Subsequently, representative MS spectra of these clusters can be compared with spectra in the MALDI-TOF MS AAB reference database, which will identify most AAB isolates. Throughout our studies we consistently noted that isolates from a single cluster represented the same species, so we confirmed that, when dealing with large number of isolates, MALDI-TOF MS is an ideal dereplication and identification tool that can be combined with the use of multiple isolation media and procedures.

8

Defining AAB in the genomic era

"I look at the term species as one arbitrarily given for the sake of convenience to a set of individuals resembling each other."

Charles Darwin
The Origin of Species

8.1. The current practice of polyphasic taxonomy of AAB

Although the taxonomy of AAB has been improved in the past few decades, classification ambiguities still exist (**Chapter 3**) (Papalexandratou *et al.*, 2009). In the frame of the present Ph.D study, we also aimed to improve the current AAB classification by means of whole-genome sequence based approaches.
Despite the arbitrary nature of a bacterial species definition, it provides a way to organize and understand the microbial world. Current AAB taxonomy is commonly the result of a polyphasic approach combining different types of data including 16S rRNA gene sequences, DNA-DNA hybridization data, DNA fingerprint data, chemotaxonomic data, and physiological and morphological information. However, the discriminatory power of 16S rRNA gene sequences is rather limited when applied to AAB species; pairwise sequence similarities range from 95.4 to 99.9 % within the genus *Acetobacter*, from 99.6 to 99.8 % within the genus *Asaia*, from 96.4 to 100 % within the genus *Gluconacetobacter*, 98.1 to 100 % within the genus *Komagataeibacter* and 98.3 to 99.6 % within the genus *Gluconobacter*. Generally speaking, when the 16S rRNA gene sequence similarity is $>$ 98.65 %, DNA-DNA hybridizations or equivalent analyses need to be performed to be able to classify isolates accurately at the species level (Kim *et al.*, 2014; Tindall *et al.*, 2010). The 70 % DNA-DNA hybridization (DDH) value is since many years considered the "gold standard" for species delineation although this method has many obvious disadvantages. Indeed, it is a labor-intensive research technique and sometimes gives discrepant results. Especially for AAB strains that produce exopolysaccharides, problems often start from the very first step in the procedure, *i.e.* the extraction of high quality genomic DNA.
Another method that is now commonly used in AAB classification is multilocus sequence analysis (MLSA). MLSA based on the three housekeeping genes *dnaK*, *groEL* and *rpoB* has been shown useful in taxonomic studies of the genera *Gluconacetobacter*, *Acetobacter* and

related taxa, and has a higher resolution than 16S rRNA gene sequencing (Cleenwerck *et al.*, 2010; Li *et al.*, 2014). Yet, there are no universal primers that can be used to analyze all AAB by means of these three genes, which is a problem that is common to many MLSA schemes (Coenye *et al.*, 2005). Moreover, for some AAB species the number of reference strains for which sequences are available is rather limited, which prevents to set a general cutoff for species delineation based on individual or concatenated sequence similarities. Furthermore, MLSA of *dnaK*, *groEL* and *rpoB* does not allow to distinguish among some closely related AAB species, such as *A. malorum* and *A. cerevisiae* (**Chapter 3**), or *As. siamensis* and *As. spathodea* (data not shown).
DNA fingerprinting methods such as $(GTG)_5$-PCR, AFLP and PCR-RFLP of the 16S-23S rRNA gene ITS are also often applied for AAB classification. These techniques are based on the generation and visualization of target DNA fragments after amplification or the digestion of amplicons with restriction enzymes (Gullo and Giudici, 2009). $(GTG)_5$-PCR was proven useful in screening a large number of strains. However, the discriminatory power of this technique is below the species level, where unique patterns were observed for most of AAB strains investigated before (De Vuyst *et al.*, 2008; Papalexandratou *et al.*, 2009), therefore, assigning unknowns to particular species may be cumbersome. Compared to $(GTG)_5$-PCR, AFLP provides better species level classification, yet the technique itself is labor-intensive and not suitable when handling large set of strains. The 16S-23S rRNA ITS analysis, combining different restriction enzymes was also proven useful for AAB screening and identification (Trček and Teuber, 2002; Trček and Barja, 2014). The selection of restriction enzymes is however crucial for 16S-23S rRNA gene ITS analysis, as different enzymes can result in patterns that are not suitable for species level identification (Sievers *et al.*, 1996).
A final obligatory component for the description of novel AAB, is the phenotypic and chemotaxonomic characterization. Phenotypic tests for AAB are often labor-intensive, time-consuming, lack discriminatory power and are regularly poorly reproducible leading to contradictory results. Upon describing *Bombella intestini* gen. nov., sp. nov., (**Chapter 6**), results for the production of acetic acid from ethanol, and

the production of acid from various carbon sources (*i.e.* L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, D-fructose, melibiose and D-mannitol) differed for *S. floricola* LMG 23170^{T} compared to results reported by Jojima *et al.* (2004). Similarly, part of the phenotypic characteristics of *G. kondonii* LMG 1367^{T} and *G. cerinus* NBRC 3267^{T} also yielded results that differed from those reported in previous studies (Spitaels *et al.*, 2014a).

The present polyphasic taxonomic practice also insists on determining chemotaxonomic characteristics of novel bacteria (Tindall *et al.*, 2010). Apart from the earlier discussed MALDI-TOF MS, the determination of respiratory quinone and whole cell fatty acid methyl ester (FAME) composition are the chemotaxonomic cell components that are most commonly analyzed in taxonomic studies of AAB. The latter requires very standardized growth conditions, *i.e.* test organisms should be streaked the same way on the same medium and cultivated at the same temperature for the same time period. Moreover, only colonies from a specific area on the agar plate should be harvested and subjected to fatty acid extraction. Very commonly cellular fatty acid analysis does not allow species level differentiation. As for the analysis of respiratory quinones, all investigated *Acetobacter* species have Q-9 as the major ubiquinone, while species of other genera possess Q-10 as the major ubiquinone.

8.2. Genomic approach towards AAB classification

With the current development of sequencing and computing technology, bacterial taxonomy is now entering the genomic era. In the past 10 years, the cost of sequencing per nucleotide has dropped about 10^2–10^3 fold (Arahal, 2014), thereby making whole genome sequencing an affordable option for many laboratories. Along with the development of sequencing technology, computing tools for subsequent analyses are emerging, making routine genome analysis in laboratory practice within reach.

As mentioned above, in current AAB taxonomy, phylogeny and species delineation rely largely on 16S rRNA gene sequencing, MLSA, DNA fingerprinting and DDH. Many of the closely related AAB species can not be distinguished or well recognized on the basis of these techniques. In **Chapter 3**, a large group of AAB strains were investigated by means of MALDI-TOF MS, MLSA, AFLP and DDH. However, *G. uchimurae* and *G. nephelii* could not be distinguished from *G. oxydans* and *G. japonicus*, respectively, whereas *A. malorum* and *A. cerevisiae* could not be well recognized either (DDH yielded variable results close to the species delineation threshold, MLSA could not distinguish them, and AFLP revealed too many clusters). In contrast, whole genome ANI values of these problematic groups were easily calculated: strains of *G. uchimurae* and *G. nephelii* showed $>$ 95 % ANI value to strains of *G. oxydans* and *G. japonicus*, respectively, and therefore *G. uchimurae* and *G. nephelii* were revealed as synonyms of the latter two species. In addition, *A. malorum* and *A. cerevisiae* were revealed to be distinct species, yet several strains proved misclassified.
In our study, the MUMmer software package was used for sequence alignment and an in-house developed python script based on JSpecies (Richter and Rossello-Mora, 2009) was used to calculate ANIm values. Alternatively, ANIb values determined using BLAST search algorithms can also be used. In the calculation of ANIb, genomic DNA of query genome sequence is first segmented into consecutive 1020 nt fragments, corresponding with the genomic DNA fragments formed during experimental DDH reactions. Subsequently, fragments of the query genome are used to search against the whole-genomic sequence of the subject genome by using BLASTn; the best matches are then saved for further analysis. Reverse searching is performed to provide reciprocal values. The ANIb value is then calculated as the mean identity of all BLASTn matches that showed more than 30 % overall sequence identity over an alignable region of at least 70 % (Goris *et al.*, 2007; Richter and Rossello-Mora, 2009). Due to the different algorithms used in ANIb and ANIm analyses, the run-time of the latter is however much shorter than that of the former at a genome scale (Arahal, 2014).
Besides ANI calculations, other genomic parameters such as the genome-

to-genome distance calculator (GGDC) or tetra-nucleotide frequency analyses can be used to calculate between-genome distances (Meier-Kolthoff *et al.*, 2013; Pride *et al.*, 2003). Furthermore, phylogenetic analyses based on single 16S rRNA gene or even several concatenated housekeeping gene sequences can now be complemented with whole genome phylogenetic analyses based on core orthologs (Chan *et al.*, 2012).

Moreover, whole genome sequences also provide a large amount of information on the microorganisms' metabolic and physiological capacities. In **Chapter 6**, the genome sequence of *Bombella intestini* LMG 28161^{T} revealed metabolic pathways and supported results from *in vitro* experiments. Moreover, its relatively small genome size, 2.03 Mb, the presence of ABC transporters and various secretion systems indicate that this strain may have adapted to its special niche as insect endosymbiont. Yet, certain phenotypic features such as growth characteristics, colony and cell morphology, are not only the result of the expression of genes but also reflect the conditions under which the genes are expressed, meaning that such complex features cannot simply be deduced from the presence or absence of specific genes (Kämpfer, 2014).

Nevertheless, the practice of polyphasic taxonomy is discouraging considering the great number of novel taxa being discovered nowadays. AAB are now being found in many new niches, such as insect guts, stone surfaces and the human environment (Crotti *et al.*, 2010; Greenberg *et al.*, 2006; Nishijima *et al.*, 2013; Tazato *et al.*, 2012). In order to learn about them more rapidly in the near future, it is necessary to compromise on a part of the laboratory practice, for instance by reducing the phenotypic and chemotaxonomic tests to a minimum. It has been proposed that novel species descriptions in the future should include a basic biological identity card that comprises a full genome sequence and a minimal description of phenotypic characteristics (Vandamme and Peeters, 2014). A five-step roadmap for species description within known genera has also been proposed recently (Thompson *et al.*, 2015). In my view, phenotypic characteristics provide certain values in taxonomy, however, for AAB species, phenotypic differentiation by means of classical biochemical test is mostly based on few characteristics

and should not be considered essential for reporting novel species. Yet, basic features, such as optimal growth medium, growth atmosphere, temperature, cell and colony morphology should be included to provide basic information to researchers to work on this organism.
As for higher taxa, genera are circumscribed mainly by their phylogenetic separation based on 16S rRNA gene sequence similarity (Yarza *et al.*, 2014). For instance, the genus *Gluconoacetobacter* was elevated from a subgenus of *Acetobacter* to the genus level based on 16S rRNA sequence analysis by Yamada in 1997, and was later named *Gluconacetobacter* (Yamada *et al.*, 1997). This genus delineation is also supported by some phenotypic or chemotaxonomic features: *Acetobacter* sp. are featured with Q-9 as major ubiquinone, are able to grow at 37 ℃ and have a DNA G+C content range from 52 to 61; in contrast, *Gluconacetobacter* has Q-10 as major ubiquinone, is unable to grow at 37 ℃ and has a DNA G+C content range from 62-67. In addition, *Acetobacter* are mostly food related and *Gluconacetobacter* are mostly plant related. Based on phylogeny, phenotypic characteristics and ecological niches, the genus *Gluconacetobacter* was however again split into two genera in 2012, *Gluconacetobacter* and *Komagataeibacter* (Yamada *et al.*, 2012). The type strains of the type species of the two genera showed 96 % sequence similarity. *Gluconacetobacter* species are motile and are equipped with peritrichous flagella, while *Komagataeibacter* species are non-motile and without any flagellation. The former produces a water soluble brown pigment when grown on glucose/yeast extract/calcium carbonate medium, while the latter does not. The former produces 2,5-diketo-D-gluconate and γ-pyrone compounds from D-glucose, while again the latter does not. *Gluconacetobacter* is plant-associated, while *Komagataeibacter* occupies more diverse niches. Although the information provided above shows that the phylogenetic differentiation is also supported by a polyphasic differentiation of these higher taxa, there are no widely accepted criteria for polyphasic differentiation of higher taxa. Also in this context studies of whole genome sequences may prove valuable as it has been suggested that the percentage of conserved proteins (POCP) could be considered a robust genomic index for establishing the genus boundary for prokaryotic groups: two species belonging to the same genus would

share at least half of their proteins (Qin *et al.*, 2014). The applicability of such novel genus differentiation parameters should however be tested on a much larger number of bacterial taxa first.
Above the genus level, 86.5 % 16S rRNA gene sequence similarity has been proposed as a general threshold for family level delineation (Yarza *et al.*, 2014). Most researchers restrict the family *Acetobacteraceae* to AAB species. However, in the 4th edition of *The Prokaryotes* (DeLong *et al.*, 2014; Komagata *et al.*, 2014) and on the list of prokaryotic names with standing in nomenclature (`http://www.bacterio.net`), the family *Acetobacteraceae* refers to both AAB and acidophilic bacteria such as *Acidiphilium*, *Acidicaldus* and *Rhodopila*. Phylogenetically, the two groups are distinct (Kersters *et al.*, 2006) : the 16S rRNA gene sequence similarity level of the *A. aceti* type strain towards the *Acidiphilium cryptum* type strain is 86.5 % (data obtained in this study), placing it at the phylogenetic threshold level for family delineation proposed by Yarza *et al.* (2014). Taking into account their distinct phenotypic characteristics, such as oxidase activity, optimum growth temperature and pH, and percentage %G+C DNA content, it seems reasonable to classify the two groups into separate families. Again, whole genome sequence based parameters may shed new light on the circumscription of higher taxa as well.

Part V.

Summary

Nederlandstalige Samenvatting

Azijnzuurbacteriën (AAB) zijn een groep voedselgerelateerde en industriële micro-organismen. Ze kunnen zowel een positieve als een negatieve rol spelen in gisting van voedsel en drank, en zijn wijdverbreid in de natuurlijke en menselijke omgeving. Sommige soorten AAB zijn opportunistische menselijke ziekteverwekkers, terwijl sommige anderen symbiotische relaties geëvolueerd hebben met insecten. Gezien hun belang in de industrie, de geneeskunde en de landbouw, is hun isolatie en snelle identificatie vereist om een beter inzicht te krijgen in deze organismen.
De identificatie van AAB op basis van een fenotypische benadering is moeilijk en vaak niet nauwkeurig. Moleculair-gebaseerde methoden zijn eenvoudiger en laten vaak een identificatie tot op speciesniveau toe. Hoewel de AAB-taxonomie via deze laatste werkwijze verbeterd is in de afgelopen twee decennia, blijven een aantal nauw verwante soorten AAB moeilijk te onderscheiden. Bovendien zijn veel van deze methoden zeer arbeidsintensief en/of tijdrovend, vooral bij de behandeling van een groot aantal AAB-isolaten. Daarom is een snelle en nauwkeurige tool voor AAB-identificatie nodig. Matrix-geassisteerde laser desorptie/ionisatie time-of-flight massaspectrometrie (MALDI-TOF MS) heeft eerder haar nut bewezen als techniek voor de identificatie van een beperkt aantal AAB species. Het primaire doel van deze studie is het ontwikkelen en evalueren van een robuuste MALDI-TOF MS-database voor de identificatie van nieuwe AAB-isolaten, en het aanpakken van bepaalde bestaande taxonomische dubbelzinnigheden middels een op volledige-genoomsequenering gebaseerde aanpak.
De MALDI-TOF MS AAB referentiegegevensbank gebouwd in de huidige studie bestaat uit ongeveer 300 goed geïdentificeerde

AAB stammen en werd verder geëvalueerd door een reeks cacaoboonfermentatieproces-isolaten en meer dan 260 slecht gekenmerkte AAB stammen uit de BCCM/LMG Bacteria Collection. Door vergelijking van de MS profielen van ongeïdentificeerde stammen met die van de referentiestammen, konden de meeste isolaten en stammen geïdentificeerd worden tot op species-niveau. De overige stammen waren ofwel nieuwe soorten van de familie, ofwel geen AAB-stammen. Tijdens de bouw van de gegevensbank werden AAB-isolaten van kefir en hommeldarmen verzameld en gederepliceerd met behulp van MALDI-TOF MS. Vervolgens werden representatieve isolaten geselecteerd en onderworpen aan identificatie met de MALDI-TOF MS-referentiedatabank. Een nieuw species *Acetobacter sicerae* sp. nov., (geïsoleerd uit kefir) en een nieuw genus *Bombella intestini* gen. nov., sp. nov. (geïsoleerd uit de krop van een hommel) werden beschreven.
De snelle ontwikkeling van de volgende generatie sequeneringsmethoden heeft volledige-genoomsequenering (WGS) betaalbaar en haalbaar gemaakt in de routine laboratoriumpraktijk. Volledige genoomsequenties bevatten taxonomische informatie en kunnen daarom worden gebruikt als solide kader voor de identificatie en classificatie van bacteriële soorten. Sommige taxonomische problemen binnen de familie van de *Acetobacteraceae* werden bevestigd door middel van MALDI-TOF MS gegevens, en werden verder onderzocht door middel van WGS-analyse. De waarde van de gemiddelde nucleotide identiteit (ANI) tussen verschillende nauw verwante soorten werd berekend. Dit onthulde de synonymie van *Gluconobacter oxydans* en *Gluconobacter uchimurae*, en *Gluconobacter japonicus* en *Gluconobacter nephelii*.
Ten laatste leverden volledige genoomsequenties een grote hoeveelheid informatie op over de metabolische en fysiologische capaciteiten van de bestudeerde microorganismen. WGS-analyse van *B. intestini* LMG 28161^{T}, een endosymbiotische AAB in hommels, werd uitgevoerd. De grootte van het genoom (2,03 Mb) van deze stam is relatief klein in vergelijking met andere AAB genomen, wat suggereert dat *B. intestini* goed aangepast is aan het spijsverteringskanaal van de hommel. De gereconstrueerde metabolische routes zijn congruent met de fenotypische kenmerken, en eerder in het kader van het onderhavige onderzoek

bepaald: de stam is in staat tot het oxideren van sucrose, D-glucose, D-fructose en D-mannitol, die allemaal aanwezig zijn in nectar of honing, terwijl ze niet in staat is ethanol of glycerol te oxideren, die ook niet beschikbaar in de hommeldarm.

English Summary

Acetic acid bacteria (AAB) are a group of food related and industrial microorganisms. They can play both a beneficial and detrimental role in food and beverage fermentation. They are widespread in natural and human environments. Some AAB species are opportunistic human pathogens, while some others evolved symbiotic relationships with insects. Given their relevance in industry, medicine and agriculture, their isolation and quick identification are required to obtain a better understanding of these organisms.

The identification of AAB based on a phenotypic approach is difficult and often not accurate. Molecular based methods are more straightforward and often allow species level identification. Although using the latter method, AAB taxonomy has been improved in the last two decades, several closely related AAB species remain difficult to differentiate. In addition, many of these methods are labor- or time-consuming, especially when handling a large number of AAB isolates. Therefore, a quick and accurate tool for AAB identification is needed. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has previously been proven useful for the identification of a limited number of AAB species. The primary goal of the present study is to develop and evaluate a robust MALDI-TOF MS database for the identification of novel AAB isolates and tackle some existing taxonomic ambiguities by means of a whole-genome sequencing based approach.

The MALDI-TOF MS AAB reference database constructed in the present study comprised approximately 300 well identified AAB

strains and was further evaluated by a set of cocoa bean fermentation isolates and more than 260 poorly characterized AAB strains from the BCCM/LMG Bacteria Collection. By comparing the MS profiles of unidentified strains to that of the reference strains, most of the isolates and strains could be identified to species level. The remaining strains were either representing novel species of the family or were no AAB. During the construction of the database, AAB isolates from kefir and bumble bee gut samples were obtained and dereplicated by MALDI-TOF MS. Subsequently, representative isolates were selected and subjected to identification using the MALDI-TOF MS reference database. A novel species *Acetobacter sicerae* sp. nov. (isolated from kefir) and a novel genus *Bombella intestini* gen. nov., sp. nov. (isolated from a bumble bee crop) were described.

The rapid development of next generation sequencing methods rendered whole-genome sequencing (WGS) affordable and feasible in routine laboratory practice. Whole genome sequences contain taxonomic information and therefore can be used to establish a solid framework for bacterial species identification and classification. Some taxonomic problems within the family *Acetobacteraceae* were confirmed through MALDI-TOF MS data and were further investigated through WGS analysis. Average nucleotide identity (ANI) value between several closely related species were calculated. This revealed the synonymy of *Gluconobacter oxydans* and *Gluconobacter uchimurae*, and of *Gluconobacter japonicus* and *Gluconobacter nephelii*.

Finally, whole genome sequences also provide a large amount of information on the microorganisms' metabolic and physiological capacities. WGS analysis of *B. intestini* LMG 28161^T, an endosymbiotic AAB in bumble bees, was carried out. The genome size (2.03 Mb) of this strain is relatively small compared to other AAB genomes, suggesting that *B. intestini* may have well adapted to the bumble bee digestive track. The reconstructed metabolic pathways are congruent with its phenotypic features determined previously and in the frame of the present study: the strain is capable of oxidizing sucrose, D-glucose,

D-fructose and D-mannitol, which are all present in nectar or honey, while it is incapable of oxidizing ethanol or glycerol, which are also not available in bumble bee gut.

Part VI.

Bibliography

Bibliography

Adachi, O., D. Moonmangmee, E. Shinagawa, H. Toyama, M. Yamada, and K. Matsushita (2003). "New quinoproteins in oxidative fermentation." In: *Biochimica et biophysica acta* 1647.1-2, pp. 10–17.

Adams, M. R. and M. O. Moss (2000). *Food Microbiology.* Royal Society of Chemistry.

Anderson, K. E., T. H. Sheehan, B. M. Mott, P. Maes, L. Snyder, M. R. Schwan, A. Walton, B. M. Jones, and V. Corby-Harris (2013). "Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (*Apis mellifera*)." In: *PLoS ONE* 8.12, e83125.

Andrés-Barrao, C., C. Benagli, M. Chappuis, R. Ortega Pérez, M. Tonolla, and F. Barja (2013). "Rapid identification of acetic acid bacteria using MALDI-TOF mass spectrometry fingerprinting". In: *Systematic and Applied Microbiology* 36.2, pp. 75–81.

Anhalt, J. P. and C. Fenselau (1975). "Identification of bacteria using mass spectrometry". In: *Analytical Chemistry* 47.2, pp. 219–225.

Arahal, D. R. (2014). "Whole-genome analyses: average nucleotide identity". In: *Bacterial Taxonomy* 41, pp. 103–122.

Asai, T., H. Iizuka, and K. Komagata (1964). "The flagellation and taxonomy of genera *Gluconobacter* and *Acetobacter* and *Acetobacter* with reference to the existence of intermediate strains". In: *Journal of General and Applied Microbiology* 10.2, pp. 95–126.

Ashbolt, N. J. and P. A. Inkerman (1990). "Acetic acid bacterial biota of the pink sugar cane mealybug, *Saccharococcus sacchari*, and Its Environs". In: *Applied and Environmental Microbiology* 56.3, pp. 707–712.

Attwood, M. M., J. P. van Dijken, and J. T. Pronk (1991). "Glucose metabolism and gluconic acid production by *Acetobacter diazotrophicus*". In: *Journal of Fermentation and Bioengineering* 72.2, pp. 101–105.

Aziz, R. K. *et al.* (2008). "The RAST Server: rapid annotations using subsystems technology." In: *BMC Genomics* 9.1, p. 75.

Azuma, Y., A. Hosoyama, M. Matsutani, N. Furuya, H. Horikawa, T. Harada, H. Hirakawa, S. Kuhara, K. Matsushita, N. Fujita, and M. Shirai (2009). "Whole-genome analyses reveal genetic instability of *Acetobacter pasteurianus*". In: *Nucleic Acids Research* 37.17, pp. 5768–5783.

Babendreier, D., D. Joller, J. Romeis, F. Bigler, and F. Widmer (2007). "Bacterial community structures in honeybee intestines and their response to two insecticidal proteins." In: *FEMS Microbiology Ecology* 59.3, pp. 600–610.

Backert, S., N. Tegtmeyer, and W. Fischer (2015). "Composition, structure and function of the *Helicobacter pylori cag* pathogenicity island encoded type IV secretion system." In: *Future Microbiology* 10.6, pp. 955–965.

Beppu, T. (1993). "Genetic organization of *Acetobacter* for acetic acid fermentation." In: *Antonie van Leeuwenhoek* 64.2, pp. 121–135.

Bernardo, K., N. Pakulat, M. Macht, O. Krut, H. Seifert, S. Fleer, F. Hünger, and M. Krönke (2002). "Identification and discrimination of *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry." In: *Proteomics* 2.6, pp. 747–753.

Bertalan, M. *et al.* (2009). "Complete genome sequence of the sugarcane nitrogen-fixing endophyte *Gluconacetobacter diazotrophicus* Pal5". In: *BMC Genomics* 10.1, p. 450.

Bielecki, S., A. Krystynowicz, M. Turkiewicz, and H. Kalinowska (2005). *Bacterial cellulose.* Ed. by E. J. Vandamme, S. De Baets, and A. Steinbüchel. Biology • Chemistry • Biotechnology • Applications. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.

Bizzini, A. and G. Greub (2010). "Matrix-assisted laser desorption ionization time-of-flight mass spectrometry, a revolution in clinical

microbial identification". In: *Clinical Microbiology and Infection* 16.11, pp. 1614–1619.

Boesch, C., J. Trček, M. Sievers, and M. Teuber (1997). "*Acetobacter intermedius*, sp. nov." In: *Systematic and Applied Microbiology* 21.2, pp. 220–229.

Bohlin, J. and E. Skjerve (2009). "Examination of genome homogeneity in prokaryotes using genomic signatures." In: *PloS One* 4.12, e8113.

Böhme, K., I. C. Fernández-No, J. Barros-Velázquez, J. M. Gallardo, B. Cañas, and P. Calo-Mata (2010). "Comparative analysis of protein extraction methods for the identification of seafood-borne pathogenic and spoilage bacteria by MALDI-TOF mass spectrometry ". In: *Analytical Methods* 2.12, pp. 1941–1947.

Böhme, K., S. Morandi, P. Cremonesi, I. C. Fernández-No, J. Barros-Velázquez, B. Castiglioni, M. Brasca, B. Cañas, and P. Calo-Mata (2012). "Characterization of *Staphylococcus aureus* strains isolated from Italian dairy products by MALDI-TOF mass fingerprinting". In: *Electrophoresis* 33.15, pp. 2355–2364.

Bossi, R. T., A. Negri, G. Tedeschi, and A. Mattevi (2002). "Structure of FAD-bound L-aspartate oxidase: insight into substrate specificity and catalysis." In: *Biochemistry* 41.9, pp. 3018–3024.

Brown, R. M. and I. M. Saxena (2007). *Cellulose: molecular and structural biology.* Selected Articles on the Synthesis, Structure, and Applications of Cellulose. Springer Science & Business Media.

Buchanan, R. E., D. H. Bergey, J. G. Holt, and E. F. Lessel (1966). *Index Bergeyana. An annotated alphabetic listing of names of the taxa of the* Bacteria.

Busse, H. J., M. Wieser, and S. Buczolits (2012). *Arthrobacter.* Chichester, UK: John Wiley & Sons, Ltd.

Cariveau, D. P., J. Elijah Powell, H. Koch, R. Winfree, and N. A. Moran (2014). "Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*)." In: *The ISME Journal* 8.12, pp. 2369–2379.

Carr, J. G. (1958). "*Acetobacter estunense* nov. spec., an addition to Frateur's ten basic species." In: *Antonie van Leeuwenhoek* 24.2, pp. 157–160.

Castro, C., I. Cleenwerck, J. Trček, R. Zuluaga, P. De Vos, G. Caro, R. Aguirre, J.-L. Putaux, and P. Gañán (2013). "*Gluconacetobacter medellinensis* sp. nov., cellulose- and non-cellulose-producing acetic acid bacteria isolated from vinegar." In: *International Journal of Systematic and Evolutionary Microbiology* 63.3, pp. 1119–1125.

Chan, J. Z.-M., M. R. Halachev, N. J. Loman, C. Constantinidou, and M. J. Pallen (2012). "Defining bacterial species in the genomic era: insights from the genus *Acinetobacter*." In: *BMC Microbiology* 12.1, p. 302.

Cheldelin, V. H., J. T. Cummins, and T. E. King (1957). "The biological oxidation of sorbitol." In: *The Journal of Biological Chemistry* 224.1, pp. 323–329.

Chouaia, B., S. Gaiarsa, E. Crotti, F. Comandatore, M. Degli Esposti, I. Ricci, A. Alma, G. Favia, C. Bandi, and D. Daffonchio (2014). "Acetic acid bacteria genomes reveal functional traits for adaptation to life in insect guts". In: *Genome Biology and Evolution* 6.4, pp. 912–920.

Clark, A. E., E. J. Kaleta, A. Arora, and D. M. Wolk (2013). "Matrix-assisted laser desorption ionization time-of-flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology". In: *Clinical Microbiology Reviews* 26.3, pp. 547–603.

Claydon, M. A., S. N. Davey, V. Edwards-Jones, and D. B. Gordon (1996). "The rapid identification of intact microorganisms using mass spectrometry". In: *Nature Biotechnology* 14.11, pp. 1584–1586.

Cleenwerck, I., K. Vandemeulebroecke, D. Janssens, and J. Swings (2002). "Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov". In: *International Journal of Systematic and Evolutionary Microbiology* 52.5, pp. 1551–1558.

Cleenwerck, I., N. Camu, K. Engelbeen, T. De Winter, K. Vandemeulebroecke, P. De Vos, and L. De Vuyst (2007). "*Acetobacter ghanensis* sp. nov., a novel acetic acid bacterium isolated from traditional heap fermentations of Ghanaian cocoa beans". In: *International Journal of Systematic and Evolutionary Microbiology* 57.7, pp. 1647–1652.

Cleenwerck, I. and P. De Vos (2008). "Polyphasic taxonomy of acetic acid bacteria: an overview of the currently applied methodology". In: *International Journal of Food Microbiology* 125.1, pp. 2–14.

Cleenwerck, I., P. De Vos, and L. De Vuyst (2010). "Phylogeny and differentiation of species of the genus *Gluconacetobacter* and related taxa based on multilocus sequence analyses of housekeeping genes and reclassification of *Acetobacter xylinus* subsp. sucrofermentans as *Gluconacetobacter sucrofermentans* (Toyosaki et al. 1996) sp. nov., comb. nov." In: *International Journal of Systematic and Evolutionary Microbiology* 60.10, pp. 2277–2283.

Cleenwerck, I., M. De Wachter, A. González, L. De Vuyst, and P. De Vos (2009). "Differentiation of species of the family *Acetobacteraceae* by AFLP DNA fingerprinting: *Gluconacetobacter kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii*". In: *International Journal of Systematic and Evolutionary Microbiology* 59.7, pp. 1771–1786.

Cleenwerck, I., A. González, N. Camu, K. Engelbeen, P. De Vos, and L. De Vuyst (2008). "*Acetobacter fabarum* sp. nov., an acetic acid bacterium from a Ghanaian cocoa bean heap fermentation". In: *International Journal of Systematic and Evolutionary Microbiology* 58.9, pp. 2180–2185.

Coenye, T., D. Gevers, Y. Van de Peer, P. Vandamme, and J. Swings (2005). "Towards a prokaryotic genomic taxonomy". In: *FEMS Microbiology Reviews* 29.2, pp. 147–167.

Conway, G. C., S. C. Smole, D. A. Sarracino, R. D. Arbeit, and P. E. Leopold (2001). "Phyloproteomics: species identification of *Enterobacteriaceae* using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry." In: *Journal of Molecular Microbiology and Biotechnology* 3.1, pp. 103–112.

Corby-Harris, V., A. C. Pontaroli, L. J. Shimkets, J. L. Bennetzen, K. E. Habel, and D. E. L. Promislow (2007). "Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*." In: *Applied and Environmental Microbiology* 73.11, pp. 3470–3479.

Corby-Harris, V., L. A. Snyder, M. R. Schwan, P. Maes, Q. S. McFrederick, and K. E. Anderson (2014). "Origin and effect of Alpha 2.2 *Acetobacteraceae* in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov." In: *Applied and Environmental Microbiology* 80.24, pp. 7460–7472.

Cox, C. R. and M. S. Gilmore (2007). "Native microbial colonization of Drosophila melanogaster and its use as a model of *Enterococcus faecalis* pathogenesis." In: *Infection and immunity* 75.4, pp. 1565–1576.

Crafack, M., M. B. Mikkelsen, S. Saerens, M. Knudsen, A. Blennow, S. Lowor, J. Takrama, J. H. Swiegers, G. B. Petersen, H. Heimdal, and D. S. Nielsen (2013). "Influencing cocoa flavour using Pichia kluyveri and Kluyveromyces marxianus in a defined mixed starter culture for cocoa fermentation". In: *International Journal of Food Microbiology* 167.1, pp. 103–116.

Crawford, R. L. (1981). *Lignin Biodegradation and Transformation.* John Wiley & Sons.

Crotti, E., C. Damiani, M. Pajoro, E. Gonella, A. Rizzi, I. Ricci, I. Negri, P. Scuppa, P. Rossi, P. Ballarini, N. Raddadi, M. Marzorati, L. Sacchi, E. Clementi, M. Genchi, M. Mandrioli, C. Bandi, G. Favia, A. Alma, and D. Daffonchio (2009). "*Asaia*, a versatile acetic acid bacterial symbiont, capable of cross-colonizing insects of phylogenetically distant genera and orders". In: *Environmental Microbiology* 11.12, pp. 3252–3264.

Crotti, E., A. Rizzi, B. Chouaia, I. Ricci, G. Favia, A. Alma, L. Sacchi, K. Bourtzis, M. Mandrioli, A. Cherif, C. Bandi, and D. Daffonchio (2010). "Acetic acid bacteria, newly emerging symbionts of insects." In: *Applied and Environmental Microbiology* 76.21, pp. 6963–6970.

Damiani, C., I. Ricci, E. Crotti, P. Rossi, A. Rizzi, P. Scuppa, F. Esposito, C. Bandi, D. Daffonchio, and G. Favia (2008). "Paternal transmission of symbiotic bacteria in malaria vectors." In: *Current Biology* 18.23, R1087–8.

Daubin, V., M. Gouy, and G. Perrière (2001). "Bacterial molecular phylogeny using supertree approach." In: *Genome Informatics. International Conference on Genome Informatics* 12, pp. 155–164.

De Bruyne, K., N. Camu, L. De Vuyst, and P. Vandamme (2010). "*Weissella fabaria* sp. nov., from a Ghanaian cocoa fermentation". In: *International Journal of Systematic and Evolutionary Microbiology* 60.9, pp. 1999–2005.

De Bruyne, K., B. Slabbinck, W. Waegeman, P. Vauterin, B. De Baets, and P. Vandamme (2011). "Bacterial species identification from MALDI-TOF mass spectra through data analysis and machine learning". In: *Systematic and Applied Microbiology* 34.1, pp. 20–29.

De Ley, J. (1961). "Comparative carbohydrate metabolism and a proposal for a phylogenetic relationship of the acetic acid bacteria". In: *Journal of general microbiology* 24.1, pp. 31–50.

De Ley, J. and J. Frateur (1974a). "Genus *Acetobacter* Beijerinck 1898, 215^{AL} ". In: *Bergeys Manual of Determinative Bacteriology*. Ed. by R. E. Buchanan and N. E. Gibbons. Baltimore: Williams & Wilkins, pp. 276–278.

De Ley, J. and J. Frateur (1974b). "Genus *Gluconobacter* Beijerinck 1898, 215^{AL} ". In: *Bergeys manual of determinative bacteriology*. Ed. by R. E. Buchanan and N. E. Gibbons. Baltimore: Williams & Wilkins, pp. 251–253.

De Muynck, C., C. S. S. Pereira, M. Naessens, S. Parmentier, W. Soetaert, and E. J. Vandamme (2007). "The genus *Gluconobacter oxydans*: comprehensive overview of biochemistry and biotechnological applications." In: *Critical Reviews in Biotechnology* 27.3, pp. 147–171.

De Vuyst, L., N. Camu, T. De Winter, K. Vandemeulebroecke, V. Van de Perre, M. Vancanneyt, P. De Vos, and I. Cleenwerck (2008). "Validation of the $(GTG)_5$-rep-PCR fingerprinting technique for rapid classification and identification of acetic acid bacteria, with a focus on isolates from Ghanaian fermented cocoa beans". In: *International Journal of Food Microbiology* 125.1, pp. 79–90.

De Vuyst, L., T. Lefeber, Z. Papalexandratou, and N. Camu (2010). "The functional role of lactic acid bacteria in cocoa bean fermentation". In: *Biotechnology of Lactic Acid Bacteria*. Oxford, UK: Wiley-Blackwell, pp. 301–325.

Dellaglio, F. (2005). "Description of *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., isolated from Italian

apple fruit". In: *International Journal of Systematic and Evolutionary Microbiology* 55.6, pp. 2365–2370.

DeLong, E. F., S. Lory, E. Stackebrandt, and F. Thompson (2014). *The Prokaryotes*. Ed. by E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt, and F. Thompson. Alphaproteobacteria and Betaproteobacteria. Berlin, Heidelberg: Springer.

DeMarco, M. L. and B. A. Ford (2013). "Beyond identification: emerging and future uses for MALDI-TOF mass spectrometry in the clinical microbiology laboratory." In: *Clinics in Laboratory Medicine* 33.3, pp. 611–628.

Deppenmeier, U., M. Hoffmeister, and C. Prust (2002). "Biochemistry and biotechnological applications of *Gluconobacter* strains." In: *Applied Microbiology and Biotechnology* 60.3, pp. 233–242.

Despeyroux, D., R. Phillpotts, and P. Watts (1996). "Electrospray mass spectrometry for detection and characterization of purified cricket paralysis virus (CrPV)". In: *Rapid Communications in Mass Spectrometry* 10.8, pp. 937–941.

Dieckmann, R., I. Graeber, I. Kaesler, U. Szewzyk, and H. von Döhren (2005). "Rapid screening and dereplication of bacterial isolates from marine sponges of the sula ridge by intact-cell-MALDI-TOF mass spectrometry (ICM-MS)." In: *Applied Microbiology and Biotechnology* 67.4, pp. 539–548.

Dieckmann, R., R. Helmuth, M. Erhard, and B. Malorny (2008). "Rapid classification and identification of *Salmonellae* at the species and subspecies levels by whole-cell matrix-assisted laser desorption ionization time-of-flight mass spectrometry". In: *Applied and Environmental Microbiology* 74.24, pp. 7767–7778.

Dufresne, C. and E. Farnworth (2000). "Tea, Kombucha, and health: a review". In: *Food Research International* 33.6, pp. 409–421.

Duim, B. and J. Wagenaar (2006). "Amplified fragment-length polymorphism and protein profiling for identification of *Campylobacter lari* subgroups." In: *Methods in Molecular Biology* 345, pp. 119–130.

Dutta, D. and R. Gachhui (2006). "Novel nitrogen-fixing *Acetobacter nitrogenifigens* sp. nov., isolated from Kombucha tea". In: *International*

Journal of Systematic and Evolutionary Microbiology 56.8, pp. 1899–1903.

Dutta, D. and R. Gachhui (2007). "Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from Kombucha tea." In: *International Journal of Systematic and Evolutionary Microbiology* 57.2, pp. 353–357.

Ebner, H. and H. F. S. Sellmer (1995). *Vinegar*. Weinheim, Germany: Wiley-VCH Verlag GmbH.

Edgar, R. C. (2004). "MUSCLE: a multiple sequence alignment method with reduced time and space complexity". In: *BMC Bioinformatics* 5, p. 113.

Ellwood, D. C. and D. W. Tempest (1972). "Influence of culture pH on the content and composition of teichoic acids in the walls of *Bacillus subtilis*." In: *Journal of General Microbiology* 73.2, pp. 395–402.

Emerson, D., L. Agulto, H. Liu, and L. Liu (2008). "Identifying and characterizing bacteria in an era of genomics and proteomics". In: *BioScience* 58.10, pp. 925–936.

Ezaki, T., Y. Hashimoto, and E. Yabuuchi (1989). "Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains". In: *International Journal of Systematic and Evolutionary Microbiology* 39.3, pp. 224–229.

Favia, G., I. Ricci, M. Marzorati, I. Negri, A. Alma, L. Sacchi, C. Bandi, and D. Daffonchio (2008). "Bacteria of the genus *Asaia*: a potential paratransgenic weapon against malaria." In: *Advances in Experimental Medicine and Biology* 627.Chapter 4, pp. 49–59.

Favia, G. *et al.* (2007). "Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector." In: *Proceedings of the National Academy of Sciences of the United States of America* 104.21, pp. 9047–9051.

Felsenstein, J. (1981). "Evolutionary trees from DNA sequences: A maximum likelihood approach". In: *Journal of Molecular Evolution* 17.6, pp. 368–376.

Felsenstein, J. (1985). "Confidence limits on phylogenies: an approach using the bootstrap". In: *Evolution* 39.4, p. 783.

Ferreira, L., F. Sánchez-Juanes, M. González-Avila, D. Cembrero-Fuciños, A. Herrero-Hernández, J. M. González-Buitrago, and J. L. Muñoz-Bellido (2010). "Direct identification of urinary tract pathogens from urine samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry." In: *Journal of Clinical Microbiology* 48.6, pp. 2110–2115.

Fournier, R., F. Wallet, B. Grandbastien, L. Dubreuil, R. Courcol, C. Neut, and R. Dessein (2012). "Chemical extraction versus direct smear for MALDI-TOF mass spectrometry identification of anaerobic bacteria." In: *Anaerobe* 18.3, pp. 294–297.

Franke, I. H., M. Fegan, C. Hayward, G. Leonard, E. Stackebrandt, and L. I. Sly (1999). "Description of *Gluconacetobacter sacchari* sp. nov., a new species of acetic acid bacterium isolated from the leaf sheath of sugar cane and from the pink sugar-cane mealy bug". In: *International Journal of Systematic and Evolutionary Microbiology* 49.4, pp. 1681–1693.

Fuentes-Ramirez, L. E., R. Bustillos-Cristales, A. Tapia-Hernandez, T. Jimenez-Salgado, E. T. Wang, E. Martinez-Romero, and J. Caballero-Mellado (2001). "Novel nitrogen-fixing acetic acid bacteria, *Gluconacetobacter johannae* sp. nov. and *Gluconacetobacter azotocaptans* sp. nov, associated with coffee plants". In: *International Journal of Systematic and Evolutionary Microbiology* 51.4, pp. 1305–1314.

Ge, X., Y. Zhao, W. Hou, W. Zhang, W. Chen, J. Wang, N. Zhao, J. Lin, W. Wang, M. Chen, Q. Wang, Y. Jiao, Z. Yuan, and X. Xiong (2013). "Complete genome sequence of the industrial strain *Gluconobacter oxydans* H24." In: *Genome Announcements* 1.1, e00003–13.

Gevers, D., F. M. Cohan, J. G. Lawrence, B. G. Spratt, T. Coenye, E. J. Feil, E. Stackebrandt, Y. Van de Peer, P. Vandamme, F. L. Thompson, and J. Swings (2005). "Re-evaluating prokaryotic species". In: *Nature Reviews Microbiology* 3.9, pp. 733–739.

Ghyselinck, J., K. Van Hoorde, B. Hoste, K. Heylen, and P. De Vos (2011). "Evaluation of MALDI-TOF MS as a tool for high-throughput

dereplication". In: *Journal of Microbiological Methods* 86.3, pp. 327–336.

Giebel, R. A., W. Fredenberg, and T. R. Sandrin (2008). "Characterization of environmental isolates of *Enterococcus* spp. by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry". In: *Water Research* 42.4-5, pp. 931–940.

Giebel, R. A., C. Worden, S. M. Rust, G. T. Kleinheinz, M. Robbins, and T. R. Sandrin (2010). "Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) applications and challenges." In: *Advances in Applied Microbiology* 71, pp. 149–184.

Gilliam, M. (2010). "Identification and roles of non-pathogenic microflora associated with honey bees". In: *FEMS Microbiology Letters*, pp. 1–10.

Gillis, M., K. Kersters, B. Hoste, D. Janssens, R. M. Kroppenstedt, M. P. Stephan, K. Teixeira, J. Dobereiner, and J. De Ley (1989). "*Acetobacter diazotrophicus* Sp. nov, a nitrogen-fixing acetic acid bacterium associated with sugarcane". In: *International Journal of Systematic Bacteriology* 39.3, pp. 361–364.

Glaeser, S. P. and P. Kämpfer (2015). "Multilocus sequence analysis (MLSA) in prokaryotic taxonomy." In: *Systematic and Applied Microbiology* 38.4, pp. 237–245.

Gonella, E., E. Crotti, A. Rizzi, M. Mandrioli, G. Favia, D. Daffonchio, and A. Alma (2012). "Horizontal transmission of the symbiotic bacterium *Asaia* sp. in the leafhopper *Scaphoideus titanus* ball (Hemiptera: Cicadellidae)." In: *BMC Microbiology* 12 Suppl 1.Suppl 1, S4.

Goris, J., K. T. Konstantinidis, J. A. Klappenbach, T. Coenye, P. Vandamme, and J. M. Tiedje (2007). "DNA-DNA hybridization values and their relationship to whole-genome sequence similarities". In: *International Journal of Systematic and Evolutionary Microbiology* 57.1, pp. 81–91.

Goris, J., K.-i. Suzuki, P. D. Vos, T. Nakase, and K. Kersters (1998). "Evaluation of a microplate DNA - DNA hybridization method compared with the initial renaturation method". In: *Canadian Journal of Microbiology* 44.12, pp. 1148–1153.

Gosselé, F. and J. Swings (1986). “Identification of *Acetobacter liquefaciens* as causal agent of pink-disease of pineapple fruit”. In: *Journal of phytopathology*.

Gosselé, F., J. Swings, K. Kersters, and J. D. LEY (1983a). “Numerical analysis of phenotypic features and protein gel electropherograms of *Gluconobacter* Asai 1935 emend. mut. char. Asai, Iizuka, and Komagata 1964”. In: *International Journal of Systematic and Evolutionary Microbiology* 33.1, pp. 65–81.

Gosselé, F., J. Swings, K. Kersters, P. Pauwels, and J. De Ley (1983b). “Numerical analysis of phenotypic features and protein gel electrophoregrams of a wide variety of *Acetobacter* strains. Proposal for the improvement of the taxonomy of the genus *Acetobacter* Beijerinck 1898, 215”. In: *Systematic and applied Microbiology* 4, pp. 338–368.

Gosselé, F., J. Swings, D. A. Mossel, and J. De Ley (1984). “Identification of *Acetobacter* strains isolated from spoiled lactic acid fermented meat food for pets”. In: *Antonie van Leeuwenhoek* 50.3, pp. 269–274.

Greenberg, D. E., S. F. Porcella, F. Stock, A. Wong, P. S. Conville, P. R. Murray, S. M. Holland, and A. M. Zelazny (2006). “*Granulibacter bethesdensis* gen. nov., sp. nov., a distinctive pathogenic acetic acid bacterium in the family *Acetobacteraceae*”. In: *International Journal of Systematic and Evolutionary Microbiology* 56.11, pp. 2609–2616.

Greenberg, D. E., S. F. Porcella, A. M. Zelazny, K. Virtaneva, D. E. Sturdevant, J. J. Kupko, K. D. Barbian, A. Babar, D. W. Dorward, and S. M. Holland (2007). “Genome sequence analysis of the emerging human pathogenic acetic acid bacterium *Granulibacter bethesdensis*”. In: *Journal of Bacteriology* 189.23, pp. 8727–8736.

Greenwalt, C. J., K. H. Steinkraus, and R. A. Ledford (2000). “Kombucha, the fermented tea: microbiology, composition, and claimed health effects.” In: *Journal of Food Protection* 63.7, pp. 976–981.

Grissa, I., G. Vergnaud, and C. Pourcel (2007). “CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats”. In: *Nucleic Acids Research* 35.Web Server, W52–W57.

Gulitz, A., J. Stadie, M. Wenning, M. A. Ehrmann, and R. F. Vogel (2011). "The microbial diversity of water kefir." In: *International Journal of Food Microbiology* 151.3, pp. 284–288.

Gullo, M. and P. Giudici (2009). "Acetic acid bacteria aaxonomy from early descriptions to molecular techniques". In: *Vinegars of the World*. Milano: Springer Milan, pp. 41–60.

Heller, D. N., R. J. Cotter, C. Fenselau, and O. M. Uy (1987). "Profiling of bacteria by fast atom bombardment mass spectrometry." In: *Analytical Chemistry* 59.23, pp. 2806–2809.

Henneberg, W. (1897). *Beiträge zür Kenntnis der Essigbakterien.* Zentbl. Bakteriol. Parasitenkd. Infektkrankh. Hyg. Abt. II.

Henneberg, W. (1906). *Zur Kenntnis der Schnellessig- und Weinessigbakterien.*

Hillenkamp, F. and M. Karas (2007). "The MALDI process and method". In: *MALDI MS*. Wiley-VCH Verlag GmbH and Co. KGaA, pp. 1–28.

Holland, R. D., J. G. Wilkes, F. Rafii, J. B. Sutherland, C. C. Persons, K. J. Voorhees, and J. O. Lay (1996). "Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry." In: *Rapid Communications in Mass Spectrometry* 10.10, pp. 1227–1232.

Holland, R. D., F. Rafii, T. M. Heinze, J. B. Sutherland, K. J. Voorhees, and J. O. Lay (2000). "Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric detection of bacterial biomarker proteins isolated from contaminated water, lettuce and cotton cloth". In: *Rapid Communications in Mass Spectrometry* 14.10, pp. 911–917.

Huang, C.-H., M.-T. Chang, L. Huang, and W.-S. Chua (2014). "Molecular discrimination and identification of *Acetobacter* genus based on the partial heat shock protein 60 gene (hsp60) sequences." In: *Journal of the Science of Food and Agriculture* 94.2, pp. 213–218.

Iino, T., R. Suzuki, Y. Kosako, M. Ohkuma, K. Komagata, and T. Uchimura (2012a). "*Acetobacter okinawensis* sp. nov., *Acetobacter papayae* sp. nov., and *Acetobacter persicus* sp. nov.; novel acetic acid bacteria isolated from stems of sugarcane, fruits, and a flower in Japan". In: *Journal of General and Applied Microbiology* 58.3, pp. 235–243.

Iino, T., R. Suzuki, N. Tanaka, Y. Kosako, M. Ohkuma, K. Komagata, and T. Uchimura (2012b). "*Gluconacetobacter kakiaceti* sp. nov., an acetic acid bacterium isolated from a traditional Japanese fruit vinegar". In: *International Journal of Systematic and Evolutionary Microbiology* 62.7, pp. 1465–1469.

Illeghems, K., L. De Vuyst, and S. Weckx (2013a). "Complete genome sequence and comparative analysis of *Acetobacter pasteurianus* 386B, a strain well-adapted to the cocoa bean fermentation ecosystem." In: *BMC Genomics* 14.1, p. 526.

Illeghems, K., L. De Vuyst, and S. Weckx (2013b). "Complete genome sequence and comparative analysis of *Acetobacter pasteurianus* 386B, a strain well-adapted to the cocoa bean fermentation ecosystem". In: *BMC Genomics* 14.1, pp. 1–1.

Jojima, Y., Y. Mihara, S. Suzuki, K. Yokozeki, S. Yamanaka, and R. Fudou (2004). "*Saccharibacter floricola* gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen." In: *International Journal of Systematic and Evolutionary Microbiology* 54.6, pp. 2263–2267.

Joshi, V. K. (1974). "Fermentative production of L-sorbose from D-sorbitol by *Acetobacter suboxydans* (vinegar isolate)." In: *Indian journal of experimental biology* 12.5, pp. 422–424.

Kämpfer, P. (2014). "Continuing importance of the "phenotype" in the genomic rra". In: *New Approaches to Prokaryotic Systematics* 41, pp. 307–320.

Kämpfer, P. and S. P. Glaeser (2012). "Prokaryotic taxonomy in the sequencing era–the polyphasic approach revisited." In: *Environmental Microbiology* 14.2, pp. 291–317.

Kanehisa, M. and S. Goto (2000). "KEGG: kyoto encyclopedia of genes and genomes." In: *Nucleic Acids Research* 28.1, pp. 27–30.

Karas, M., D. Bachmann, U. Bahr, and F. Hillenkamp (1987). "Matrix-assisted ultraviolet laser desorption of non-volatile compounds". In: *International Journal of Mass Spectrometry and Ion Processes* 78, pp. 53–68.

Karlin, S. and C. Burge (1995). "Dinucleotide relative abundance rxtremes - a genomic signature". In: *Trends in Genetics* 11.7, pp. 283–290.

Karlin, S., J. Mrazek, and A. M. Campbell (1997). "Compositional biases of bacterial genomes and evolutionary implications." In: *Journal of Bacteriology* 179.12, pp. 3899–3913.

Katsura, K., H. Kawasaki, W. Potacharoen, S. Saono, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata (2001). "*Asaia siamensis* sp. nov., an acetic acid bacterium in the *alpha-proteobacteria*." In: *International Journal of Systematic and Evolutionary Microbiology* 51.2, pp. 559–563.

Kern, C. C., J. C. Usbeck, R. F. Vogel, and J. Behr (2013). "Optimization of Matrix-Assisted-Laser-Desorption-Ionization-Time-Of-Flight Mass Spectrometry for the identification of bacterial contaminants in beverages." In: *Journal of Microbiological Methods* 93.3, pp. 185–191.

Kersters, K., W. A. Wood, and J. Deley (1965). "Polyol dehydrogenase of *Gluconobacter oxydans*." In: *The Journal of biological chemistry* 240, pp. 965–974.

Kersters, K., P. Lisdiyanti, K. Komagata, and J. Swings (2006). "The Family *Acetobacteraceae*: The Genera *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*". In: *The Prokaryotes*. New York, NY: Springer New York, pp. 163–200.

Kim, M., H. S. Oh, S. C. Park, and J. Chun (2014). "Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes". In: *International Journal of Systematic and Evolutionary Microbiology* 64.2, pp. 346–351.

Klenk, H.-P. and M. Göker (2010). "En route to a genome-based classification of Archaea and Bacteria?" In: *Systematic and Applied Microbiology* 33.4, pp. 175–182.

Koch, H., D. P. Abrol, J. Li, and P. Schmid-Hempel (2013). "Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees". In: *Molecular Ecology* 22.7, pp. 2028–2044.

Koch, H. and P. Schmid-Hempel (2011). "Bacterial communities in central european bumblebees: low diversity and high specificity". In: *Microbial Ecology* 62.1, pp. 121–133.

Komagata, K., T. Iino, and Y. Yamada (2014). "The Family *Acetobacteraceae*". In: *The Prokaryotes*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 3–78.

Kommanee, J., S. Tanasupawat, P. Yukphan, T. Malimas, Y. Muramatsu, Y. Nakagawa, and Y. Yamada (2010). "*Asaia spathodeae* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*." In: *Journal of General and Applied Microbiology* 56.1, pp. 81–87.

Kommanee, J., S. Tanasupwawat, P. Yukphan, T. Malimas, Y. Muramatsu, Y. Nakagawa, and Y. Yamada (2011). "*Gluconobacter nephelii* sp. nov., an acetic acid bacterium in the class *Alphaproteobacteria*." In: *International Journal of Systematic and Evolutionary Microbiology* 61.9, pp. 2117–2122.

Konstantinidis, K. T., A. Ramette, and J. M. Tiedje (2006). "The bacterial species definition in the genomic era". In: *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 361.1475, pp. 1929–1940.

Konstantinidis, K. T. and J. M. Tiedje (2005). "Genomic insights that advance the species definition for prokaryotes". In: *Proceedings of the National Academy of Sciences of the United States of America* 102.7, pp. 2567–2572.

Kounatidis, I., E. Crotti, P. Sapountzis, L. Sacchi, A. Rizzi, B. Chouaia, C. Bandi, A. Alma, D. Daffonchio, P. Mavragani-Tsipidou, and K. Bourtzis (2009). "*Acetobacter tropicalis* is a major symbiont of the olive fruit fly (*Bactrocera oleae*)." In: *Applied and Environmental Microbiology* 75.10, pp. 3281–3288.

Krishnamurthy, T. and P. L. Ross (1996). "Rapid identification of bacteria by direct matrix-assisted laser desorption/ionization mass spectrometric analysis of whole cells". In: *Rapid Communications in Mass Spectrometry* 10.15, pp. 1992–1996.

Kubiak, K., M. Kurzawa, M. Jędrzejczak-Krzepkowska, K. Ludwicka, M. Krawczyk, A. Migdalski, M. M. Kacprzak, D. Loska, A. Krystynowicz, and S. Bielecki (2014). "Complete genome sequence of *Gluconaceto-*

bacter xylinus E25 strain–valuable and effective producer of bacterial nanocellulose." In: *Journal of Biotechnology* 176, pp. 18–19.

Kurtz, S., A. Phillippy, A. L. Delcher, M. Smoot, M. Shumway, C. Antonescu, and S. L. Salzberg (2004). "Versatile and open software for comparing large genomes." In: *Genome Biology* 5.2, R12.

Kwong, W. K., P. Engel, H. Koch, and N. A. Moran (2014). "Genomics and host specialization of honey bee and bumble bee gut symbionts." In: *Proceedings of the National Academy of Sciences of the United States of America* 111.31, pp. 11509–11514.

La Scola, B. and D. Raoult (2009). "Direct identification of bacteria in positive blood culture bottles by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry". In: *PLoS ONE* 4.11, e8041.

Lambert, B., K. Kersters, F. Gosselé, J. Swings, and J. De Ley (1981). "Gluconobacters from honey bees." In: *Antonie van Leeuwenhoek* 47.2, pp. 147–157.

Lapage, S. P., P. Sneath, E. F. Lessel, V. Skerman, H. Seeliger, and W. A. Clark (1992). *International code of nomenclature of bacteria: Bacteriological Code, 1990 Revision*. Washington (DC): ASM Press.

Laureys, D. and L. De Vuyst (2014). "Microbial species diversity, community dynamics, and metabolite kinetics of water kefir fermentation." In: *Applied and Environmental Microbiology* 80.8, pp. 2564–2572.

Leisinger, T., W. Jäggi, P. Weber, and L. Ettlinger (1967). "Spontaneous mutations in acetic acid bacteria. II. The meaning of mutations in systematics." In: *Archiv für Mikrobiologie* 57.1, pp. 76–92.

Li, L., 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., an acetic acid bacterium isolated from bumble bee crop." In: *International Journal of Systematic and Evolutionary Microbiology* 65.1, pp. 267–273.

Li, L., A. D. Wieme, F. Spitaels, T. Balzarini, O. C. Nunes, C. M. Manaia, A. Van Landschoot, L. De Vuyst, I. Cleenwerck, and P. A. Vandamme (2014). "*Acetobacter sicerae* sp. nov., isolated from cider and kefir and identification of *Acetobacter* species by *dnaK*, *groEL*

and *rpoB* sequence analysis". In: *International Journal of Systematic and Evolutionary Microbiology* 64.7, pp. 2407–2415.

Lisdiyanti, P. (2002). "*Kozakia baliensis* gen. nov., sp. nov., a novel acetic acid bacterium in the *alpha-Proteobacteria*". In: *International Journal of Systematic and Evolutionary Microbiology* 52.3, pp. 813–818.

Lisdiyanti, P. (2006). "Reclassification of *Gluconacetobacter hansenii* strains and proposals of *Gluconacetobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov." In: *International Journal of Systematic and Evolutionary Microbiology* 56.9, pp. 2101–2111.

Lisdiyanti, P., K. Katsura, W. Potacharoen, R. R. Navarro, Y. Yamada, T. Uchimura, and K. Komagata (2003). "Diversity of acetic acid bacteria in Indonesia, Thailand, and the Philippines". In: *Microbiology and Culture Collections* 19.2, pp. 91–99.

Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata (2000). "Systematic study of the genus *Acetobacter* with descriptions of *Acetobacter indonesiensis* sp. nov., *Acetobacter tropicalis* sp. nov., *Acetobacter orleanensis* (Henneberg 1906) comb. nov., *Acetobacter lovaniensis* (Frateur 1950) comb. nov., and *Acetobacter estunensis* (Carr 1958) comb. nov." In: *Journal of General and Applied Microbiology* 46.3, pp. 147–165.

Lisdiyanti, P., H. Kawasaki, T. Seki, Y. Yamada, T. Uchimura, and K. Komagata (2001). "Identification of *Acetobacter* strains isolated from Indonesian sources, and proposals of *Acetobacter syzygii* sp. nov., *Acetobacter cibinongensis* sp. nov., and *Acetobacter orientalis* sp. nov." In: *Journal of General and Applied Microbiology* 47.3, pp. 119–131.

Liyanage, R. and J. O. Lay (2006). "An Introduction to MALDI-TOF MS". In: *Identification of Microorganisms by Mass Spectrometry*. John Wiley and Sons, Inc., pp. 39–60.

Loganathan, P. and S. Nair (2004). "*Swaminathania salitolerans* gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate-solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka)." In: *International Journal of Systematic and Evolutionary Microbiology* 54.4, pp. 1185–1190.

Lohaus, G. and M. Schwerdtfeger (2014). "Comparison of sugars, iridoid glycosides and amino acids in nectar and phloem sap of *Maurandya barclayana*, *Lophospermum erubescens*, and *Brassica napus*". In: *PLoS ONE* 9.1, e87689.

Low, H. H., F. Gubellini, A. Rivera-Calzada, N. Braun, S. Connery, A. Dujeancourt, F. Lu, A. Redzej, R. Fronzes, E. V. Orlova, and G. Waksman (2014). "Structure of a type IV secretion system." In: *Nature* 508.7497, pp. 550–553.

Malimas, T., P. Yukphan, M. Takahashi, Y. Muramatsu, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2008a). "*Gluconobacter sphaericus* (Ameyama 1975) comb. nov., a brown pigment-producing acetic acid bacterium in the *alpha-Proteobacteria*." In: *Journal of General and Applied Microbiology* 54.4, pp. 211–220.

Malimas, T., W. Chaipitakchonlatarn, H. Thi Lan Vu, P. Yukphan, Y. Muramatsu, S. Tanasupawat, W. Potacharoen, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2013). "*Swingsia samuiensis* gen. nov., sp. nov., an osmotolerant acetic acid bacterium in the *alpha-Proteobacteria*". In: *Journal of General and Applied Microbiology* 59.5, pp. 375–384.

Malimas, T., P. Yukphan, T. Lundaa, Y. Muramatsu, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, K.-i. Suzuki, M. Tanticharoen, and Y. Yamada (2009a). "*Gluconobacter kanchanaburiensis* sp. nov., a brown pigment-producing acetic acid bacterium for Thai isolates in the *alpha-Proteobacteria*". In: *Journal of General and Applied Microbiology* 55.3, pp. 247–254.

Malimas, T., P. Yukphan, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2007). "*Gluconobacter kondonii* sp nov., an acetic acid bacterium in the *alpha-Proteobacteria*". In: *Journal of General and Applied Microbiology* 53.5, pp. 301–307.

Malimas, T., P. Yukphan, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2008b). "*Asaia lannaensis* sp. nov., a new acetic acid bacterium in the

alpha-Proteobacteria." In: *Bioscience, Biotechnology and Biochemistry* 72.3, pp. 666–671.

Malimas, T., P. Yukphan, M. Takahashi, Y. Muramatsu, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2008c). "*Gluconobacter roseus* (ex Asai 1935) sp. nov., nom. rev., a pink-colored acetic acid bacterium in the *alpha-Proteobacteria*". In: *Journal of General and Applied Microbiology* 54.2, pp. 119–125.

Malimas, T., P. Yukphan, M. Takahashi, Y. Muramatsu, M. Kaneyasu, W. Potacharoen, S. Tanasupwat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2009b). "*Gluconobacter japonicus* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria.*" In: *International Journal of Systematic and Evolutionary Microbiology* 59.3, pp. 466–471.

Marsh, A. J., O. O'Sullivan, C. Hill, R. P. Ross, and P. D. Cotter (2013). "Sequence-based analysis of the microbial composition of water kefir from multiple sources". In: *FEMS Microbiology Letters* 348.1, pp. 79–85.

Marsh, A. J., O. O'Sullivan, C. Hill, R. P. Ross, and P. D. Cotter (2014). "Sequence-based analysis of the bacterial and fungal compositions of multiple kombucha (tea fungus) samples". In: *Food Microbiology* 38.C, pp. 171–178.

Martinson, V. G., B. N. Danforth, R. L. Minckley, O. Rueppell, S. Tingek, and N. A. Moran (2011). "A simple and distinctive microbiota associated with honey bees and bumble bees". In: *Molecular Ecology* 20.3, pp. 619–628.

Marzorati, M., A. Alma, L. Sacchi, M. Pajoro, S. Palermo, L. Brusetti, N. Raddadi, A. Balloi, R. Tedeschi, E. Clementi, S. Corona, F. Quaglino, P. A. Bianco, T. Beninati, C. Bandi, and D. Daffonchio (2006). "A novel *Bacteroidetes* symbiont is localized in *Scaphoideus titanus*, the insect vector of Flavescence dorée in *Vitis vinifera.*" In: *Applied and environmental microbiology* 72.2, pp. 1467–1475.

Mason, L. M. and G. W. Claus (1989). "Phenotypic characteristics correlated with deoxyribonucleic acid sequence similarities for three species of *Gluconobacter: G. oxydans* (Henneberg 1897) De Ley 1961,

G. frateurii sp. nov., and *G. asaii* sp. nov." In: *International Journal of Systematic Bacteriology* 39.2, pp. 174–184.

Matalon, Y., N. Katzir, Y. Gottlieb, V. Portnoy, and E. Zchori-Fein (2007). "*Cardinium* in *Plagiomerus diaspidis* (Hymenoptera: Encyrtidae)." In: *Journal of invertebrate pathology* 96.2, pp. 106–108.

Matsushita, K., H. Toyama, and O. Adachi (1994). "Respiratory chains and bioenergetics of acetic acid bacteria". In: *Advances in Microbial Physiology*. Vol. 36. Elsevier, pp. 247–301.

Matsushita, K., H. Toyama, and O. Adachi (2004). "Respiratory chains in acetic acid bacteria: membranebound periplasmic sugar and alcohol respirations". In: *Respiration in* Archaea *and* Bacteria. Dordrecht: Springer Netherlands, pp. 81–99.

Matsutani, M., H. Hirakawa, N. Saichana, W. Soemphol, T. Yakushi, and K. Matsushita (2011). "Genome-wide phylogenetic analysis of differences in thermotolerance among closely related *Acetobacter pasteurianus* strains". In: *Microbiology* 158.1, pp. 229–239.

Matsutani, M., K. Fukushima, C. Kayama, M. Arimitsu, H. Hirakawa, H. Toyama, O. Adachi, T. Yakushi, and K. Matsushita (2014). "Replacement of a terminal cytochrome c oxidase by ubiquinol oxidase during the evolution of acetic acid bacteria." In: *Biochimica et Biophysica Acta* 1837.10, pp. 1810–1820.

Matsutani, M., H. Hirakawa, T. Yakushi, and K. Matsushita (2010). "Genome-wide phylogenetic analysis of *Gluconobacter*, *Acetobacter*, and *Gluconacetobacter*". In: *FEMS Microbiology Letters* 315.2, pp. 122–128.

Matsutani, M., M. Nishikura, N. Saichana, T. Hatano, U. Masud-Tippayasak, G. Theergool, T. Yakushi, and K. Matsushita (2013). "Adaptive mutation of *Acetobacter pasteurianus* SKU1108 enhances acetic acid fermentation ability at high temperature." In: *Journal of Biotechnology* 165.2, pp. 109–119.

Mazza, S. and Y. Murooka (2009). "Vinegars through the ages". In: *Vinegars of the World*, pp. 17–40.

Mazzeo, M. F., A. Sorrentino, M. Gaita, G. Cacace, M. Di Stasio, A. Facchiano, G. Comi, A. Malorni, and R. A. Siciliano (2006). "Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

for the discrimination of food-borne microorganisms." In: *Applied and Environmental Microbiology* 72.2, pp. 1180–1189.

Meier-Kolthoff, J. P., A. F. Auch, H.-P. Klenk, and M. Göker (2013). "Genome sequence-based species delimitation with confidence intervals and improved distance functions." In: *BMC Bioinformatics* 14, p. 60.

Mesbah, M., U. Premachandran, and B. Whitman William (1989). "Precise Measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography". In: *International Journal of Systematic and Evolutionary Microbiology* 39.2, pp. 159–167.

Meyer, F. (2003). "GenDB–an open source genome annotation system for prokaryote genomes". In: *Nucleic Acids Research* 31.8, pp. 2187–2195.

Micales, B. K., J. L. Johnson, and G. W. Claus (1985). "Deoxyribonucleic acid homologies among organisms in the genus *Gluconobacter*". In: *International Journal of Systematic Microbiology* 35.1, pp. 79–85.

Millet, V. and A. Lonvaud-Funel (2000). "The viable but non-culturable state of wine micro-organisms during storage". In: *Letters in Applied Microbiology* 30.2, pp. 136–141.

Moens, F., T. Lefeber, and L. De Vuyst (2014). "Oxidation of metabolites highlights the microbial interactions and role of *Acetobacter pasteurianus* during cocoa bean fermentation". In: *Applied and Environmental Microbiology* 80.6, pp. 1848–1857.

Mohr, K. I. and C. C. Tebbe (2006). "Diversity and phylotype consistency of bacteria in the guts of three bee species (*Apoidea*) at an oilseed rape field". In: *Environmental Microbiology* 8.2, pp. 258–272.

Mohr, K. I. and C. C. Tebbe (2007). "Field study results on the probability and risk of a horizontal gene transfer from transgenic herbicide-resistant oilseed rape pollen to gut bacteria of bees". In: *Applied Microbiology and Biotechnology* 75.3, pp. 573–582.

Moran, N. A. (2006). "Symbiosis." In: *Current biology : CB* 16.20, R866–71.

Mozzi, F., R. R. Raya, and G. M. Vignolo (2010). *Biotechnology of Lactic Acid Bacteria.* Novel Applications. John Wiley & Sons.

Muthukumarasamy, R., I. Cleenwerck, G. Revathi, M. Vadivelu, D. Janssens, B. Hoste, K. Ui Gum, K.-D. Park, C. Young Son, T. Sa,

and J. Caballero-Mellado (2005). "Natural association of *Gluconacetobacter diazotrophicus* and diazotrophic *Acetobacter peroxydans* with wetland rice". In: *Systematic and Applied Microbiology* 28.3, pp. 277–286.

Nakamura, S., T. Nakaya, and T. Iida (2011). "Metagenomic analysis of bacterial infections by means of high-throughput DNA sequencing." In: *Experimental Biology and Medicine* 236.8, pp. 968–971.

Navarro, R. R., T. Uchimura, and K. Komagata (1999). "Taxonomic heterogeneity of strains comprising *Gluconacetobacter hansenii*". In: *Journal of General and Applied Microbiology* 45.6, pp. 295–300.

Ndoye, B., I. Cleenwerck, K. Engelbeen, R. Dubois-Dauphin, A. T. Guiro, S. Van Trappen, A. Willems, and P. Thonart (2007). "*Acetobacter senegalensis* sp. nov., a thermotolerant acetic acid bacterium isolated in Senegal (sub-Saharan Africa) from mango fruit (*Mangifera indica* L.)." In: *International Journal of Systematic and Evolutionary Microbiology* 57.7, pp. 1576–1581.

Nei, M. and S. Kumar (2000). *Molecular evolution and phylogenetics*. New York: Oxford University Press.

Neve, H. and K. J. Heller (2002). "The microflora of water kefir: A glance by scanning electron microscopy". In: *Kieler Milchwirtschaftliche Forschungsberichte* 54.4, pp. 337–349.

Nicolaou, N., Y. Xu, and R. Goodacre (2012). "Detection and quantification of bacterial spoilage in milk and pork meat using MALDI-TOF MS and multivariate analysis." In: *Analytical Chemistry* 84.14, pp. 5951–5958.

Nilsson, A. I., S. Koskiniemi, S. Eriksson, E. Kugelberg, J. Hinton, and D. I. Andersson (2005). "Bacterial genome size reduction by experimental evolution". In: *Proceedings of the National Academy of Sciences of the United States of America* 102.34, pp. 12112–12116.

Nishijima, M., N. Tazato, Y. Handa, J. Tomita, R. Kigawa, C. Sano, and J. Sugiyama (2013). "*Gluconacetobacter tumulisoli* sp. nov., *Gluconacetobacter takamatsuzukensis* sp. nov., and *Gluconacetobacter aggeris* sp. nov., isolated from Takamatsuzuka tumulus samples before and during the dismantling work in 2007". In: *International*

Journal of Systematic and Evolutionary Microbiology 63.11, pp. 3981–3988.

Nomura, F. (2015). "Proteome-based bacterial identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS): A revolutionary shift in clinical diagnostic microbiology." In: *Biochimica et biophysica acta* 1854.6, pp. 528–537.

Ochoa, M. L. and P. B. Harrington (2005). "Immunomagnetic isolation of enterohemorrhagic *Escherichia coli* O157:H7 from ground beef and identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and database searches." In: *Analytical chemistry* 77.16, pp. 5258–5267.

Ogino, H., Y. Azuma, A. Hosoyama, H. Nakazawa, M. Matsutani, A. Hasegawa, K.-i. Otsuyama, K. Matsushita, N. Fujita, and M. Shirai (2011). "Complete genome sequence of NBRC 3288, a unique cellulose-nonproducing strain of *Gluconacetobacter xylinus* isolated from vinegar." In: *Journal of Bacteriology* 193.24, pp. 6997–6998.

Olive, D. M. and P. Bean (1999). "Principles and applications of methods for DNA-based typing of microbial organisms". In: *Journal of Clinical Microbiology* 37.6, pp. 1661–1669.

Olofsson, T. C. and A. Vásquez (2009). "Phylogenetic comparison of bacteria isolated from the honey stomachs of honey bees *Apis mellifera* and bumble bees *Bombus* spp." In: *Journal of Apicultural Research* 48.4, pp. 233–237.

Olofsson, T. C. and A. Vásquez (2008). "Detection and Identification of a Novel Lactic Acid Bacterial Flora Within the Honey Stomach of the Honeybee *Apis mellifera*". In: *Current Microbiology* 57.4, pp. 356–363.

Papaexandratou, Z., G. Vrancken, K. De Bruyne, P. Vandamme, and L. De Vuyst (2011). "Spontaneous organic cocoa bean box fermentations in Brazil are characterized by a restricted species diversity of lactic acid bacteria and acetic acid bacteria". In: *Food Microbiology* 28, pp. 1326–1338.

Papalexandratou, Z. (2011). *Species Diversity, Community Dynamics, and Metabolite Kinetics of the spontaneous cocoa bean fermentation*

process worldwide. Brussels: Research Group of Industrial Microbiology and Food Biotechnology.

Papalexandratou, Z., I. Cleenwerck, P. De Vos, and L. De Vuyst (2009). "(GTG)$_5$-PCR reference framework for acetic acid bacteria". In: *FEMS Microbiology Letters* 301.1, pp. 44–49.

Papalexandratou, Z. and L. De Vuyst (2011). "Assessment of the yeast species composition of cocoa bean fermentations in different cocoa-producing regions using denaturing gradient gel electrophoresis." In: *FEMS Yeast Research* 11.7, pp. 564–574.

Papalexandratou, Z., T. Lefeber, B. Bahrim, O. S. Lee, H.-M. Daniel, and L. De Vuyst (2013). "*Hanseniaspora opuntiae*, *Saccharomyces cerevisiae*, *Lactobacillus fermentum*, and *Acetobacter pasteurianus* predominate during well-performed Malaysian cocoa bean box fermentations, underlining the importance of these microbial species for a successful cocoa bean fermentation process". In: *Food Microbiology* 35.2, pp. 73–85.

Praet, J., I. Meeus, M. Cnockaert, M. Aerts, G. Smagghe, and P. Vandamme (2015). "*Bifidobacterium commune* sp. nov. isolated from the bumble bee gut". In: *Antonie van Leeuwenhoek* 107.5, pp. 1307–1313.

Pride, D. T., R. J. Meinersmann, T. M. Wassenaar, and M. J. Blaser (2003). "Evolutionary implications of microbial genome tetranucleotide frequency biases." In: *Genome Research* 13.2, pp. 145–158.

Prust, C., M. Hoffmeister, H. Liesegang, A. Wiezer, W. F. Fricke, A. Ehrenreich, G. Gottschalk, and U. Deppenmeier (2005). "Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*". In: *Nature Biotechnology* 23.2, pp. 195–200.

Qin, Q.-L., B.-B. Xie, X.-Y. Zhang, X.-L. Chen, B.-C. Zhou, J. Zhou, A. Oren, and Y.-Z. Zhang (2014). "A proposed genus boundary for the prokaryotes based on genomic insights." In: *Journal of Bacteriology* 196.12, pp. 2210–2215.

Quast, C., E. Pruesse, P. Yilmaz, J. Gerken, T. Schweer, P. Yarza, J. Peplies, and F. O. Glöckner (2013). "The SILVA ribosomal RNA gene database project: improved data processing and web-based tools". In: *Nucleic Acids Research* 41.Database issue, pp. D590–6.

Ramirez-Bahena, M. H., C. Tejedor, I. Martin, E. Velazquez, and A. Peix (2013). "*Endobacter medicaginis* gen. nov., sp. nov., isolated from alfalfa nodules in an acidic soil". In: *International Journal of Systematic and Evolutionary Microbiology* 63.5, pp. 1760–1765.

Raspor, P. and D. Goranovič (2008). "Biotechnological applications of acetic acid bacteria". In: *Critical Reviews in Biotechnology* 28.2, pp. 101–124.

Ren, C., P. Webster, S. E. Finkel, and J. Tower (2007). "Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off". In: *Cell Metabolism* 6.2, pp. 144–152.

Richter, M. and R. Rossello-Mora (2009). "Shifting the genomic gold standard for the prokaryotic species definition". In: *Proceedings of the National Academy of Sciences of the United States of America* 106.45, pp. 19126–19131.

Robbins, M., R. A. Giebel, T. Sandrin, and G. Kleinheinz (2007). "Matrix assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) analysis of Sunset Park *Escherichia coli* isolates". In: *Great Lakes Beach Association/State of the Lake Annual Meeting, November*, pp. 3–5.

Robinson, C. J., P. Schloss, Y. Ramos, K. Raffa, and J. Handelsman (2010). "Robustness of the bacterial community in the cabbage white butterfly larval midgut". In: *Microbial Ecology* 59.2, pp. 199–211.

Roh, S. W., Y.-D. Nam, H.-W. Chang, K.-H. Kim, M.-S. Kim, J.-H. Ryu, S.-H. Kim, W.-J. Lee, and J.-W. Bae (2008). "Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in *Drosophila melanogaster*". In: *Applied and Environmental Microbiology* 74.20, pp. 6171–6177.

Ross, P., R. Mayer, and M. Benziman (1991). "Cellulose biosynthesis and function in bacteria." In: *Microbiological Reviews* 55.1, pp. 35–58.

Ruelle, V., B. El Moualij, W. Zorzi, P. Ledent, and E. D. Pauw (2004). "Rapid identification of environmental bacterial strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry." In: *Rapid Communications in Mass Spectrometry* 18.18, pp. 2013–2019.

Ruiz-Moyano, S., N. Tao, M. A. Underwood, and D. A. Mills (2012). "Rapid discrimination of Bifidobacterium animalis subspecies by matrix-assisted laser desorption ionization time-of-flight mass spectrometry." In: *Food Microbiology* 30.2, pp. 432–437.

Ryu, J.-H., S.-H. Kim, H.-Y. Lee, J. Y. Bai, Y.-D. Nam, J.-W. Bae, D. G. Lee, S. C. Shin, E.-M. Ha, and W.-J. Lee (2008). "Innate immune homeostasis by the homeobox gene *Caudal* and commensal-gut mutualism in *Drosophila*". In: *Science* 319.5864, pp. 777–782.

Saitou, N. and M. Nei (1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees". In: *Molecular Biology and Evolution* 4.4, pp. 406–425.

Sakurai, K., H. Arai, M. Ishii, and Y. Igarashi (2011). "Transcriptome response to different carbon sources in *Acetobacter aceti*". In: *Microbiology* 157.3, pp. 899–910.

Sakurai, K., S. Yamazaki, M. Ishii, Y. Igarashi, and H. Arai (2013). "Role of the glyoxylate pathway in acetic acid production by *Acetobacter aceti*." In: *Journal of Bioscience and Bioengineering* 115.1, pp. 32–36.

Sandrin, T. R. and P. A. Demirev (2014). "Using Mass Spectrometry to identify and characterize bacteria". In: *Microbe Magazine* 9.1, pp. 23–29.

Sandrin, T. R., J. E. Goldstein, and S. Schumaker (2013). "MALDI-TOF MS profiling of bacteria at the strain level: a review." In: *Mass Spectrometry Reviews* 32.3, pp. 188–217.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber (2009). "Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities". In: *Applied and Environmental Microbiology* 75.23, pp. 7537–7541.

Schuller, G., C. Hertel, and W. P. Hammes (2000). "*Gluconacetobacter entanii* sp. nov., isolated from submerged high-acid industrial vinegar fermentations". In: *International Journal of Systematic and Evolutionary Microbiology* 50, pp. 2013–2020.

Schulthess, B., K. Brodner, G. V. Bloemberg, R. Zbinden, E. C. Böttger, and M. Hombach (2013). “Identification of Gram-positive cocci by use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry: comparison of different preparation methods and implementation of a practical algorithm for routine diagnostics.” In: *Journal of Clinical Microbiology* 51.6, pp. 1834–1840.

Schumann, P. and T. Maier (2014). *MALDI-TOF mass spectrometry applied to classification and identification of bacteria*. 1st ed. Vol. 41. Elsevier Ltd.

Schumann, P. and R. Pukall (2013). “The discriminatory power of ribotyping as automatable technique for differentiation of bacteria.” In: *Systematic and Applied Microbiology* 36.6, pp. 369–375.

Schwan, R. F. (1998). “Cocoa fermentations conducted with a defined microbial cocktail inoculum.” In: *Applied and Environmental Microbiology* 64.4, pp. 1477–1483.

Schwan, R. F. and A. E. Wheals (2004). “The microbiology of cocoa fermentation and its role in chocolate quality”. In: *Critical Reviews in Food Science and Nutrition* 44.4, pp. 205–221.

Segawa, S., S. Sawai, S. Murata, M. Nishimura, M. Beppu, K. Sogawa, M. Watanabe, M. Satoh, T. Matsutani, M. Kobayashi, Y. Iwadate, S. Kuwabara, N. Saeki, and F. Nomura (2014). “Direct application of MALDI-TOF mass spectrometry to cerebrospinal fluid for rapid pathogen identification in a patient with bacterial meningitis.” In: *Clinica chimica acta; international journal of clinical chemistry* 435, pp. 59–61.

Seng, P., M. Drancourt, F. Gouriet, B. La Scola, P.-E. Fournier, J. M. Rolain, and D. Raoult (2009). “Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.” In: *Clinical Infectious Diseases* 49.4, pp. 543–551.

Servin-Garciduenas, L. E., A. Sanchez-Quinto, and E. Martinez-Romero (2014). “Draft genome sequence of *Commensalibacter papalotli* MX01, a symbiont identified from the guts of overwintering monarch butterflies”. In: *Genome Announcements* 2.2, pages.

Shimwell, J. L. and J. G. Carr (1958). “Old and new cellulose-producing *Acetobacter* species”. In: *Journal of the Institute of Brewing* 64, pp. 477–484.

Siegrist, T. J., P. D. Anderson, W. H. Huen, G. T. Kleinheinz, C. M. McDermott, and T. R. Sandrin (2007). “Discrimination and characterization of environmental strains of *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).” In: *Journal of Microbiological Methods* 68.3, pp. 554–562.

Sievers, M., L. Alonso, S. Gianotti, C. Boesch, and M. Teuber (1996). “16S-23S ribosomal RNA spacer regions of *Acetobacter europaeus* and *A. xylinum*, tRNA genes and antitermination sequences.” In: *FEMS Microbiology Letters* 142.1, pp. 43–48.

Sievers, M., S. Sellmer, and M. Teuber (1992). “*Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in central Europe”. In: *Systematic and Applied Microbiology* 15.3, pp. 386–392.

Silva, L. R., I. Cleenwerck, R. Rivas, J. Swings, M. E. Trujillo, A. Willems, and E. Velázquez (2006). “*Acetobacter oeni* sp. nov., isolated from spoiled red wine”. In: *International Journal of Systematic and Evolutionary Microbiology* 56.1, pp. 21–24.

Slapšak, N., I. Cleenwerck, P. De Vos, and J. Trček (2013). “*Gluconacetobacter maltaceti* sp. nov., a novel vinegar producing acetic acid bacterium.” In: *Systematic and Applied Microbiology* 36.1, pp. 17–21.

Snauwaert, I., Z. Papalexandratou, L. De Vuyst, and P. Vandamme (2013). “Characterization of strains of *Weissella fabalis* sp. nov. and *Fructobacillus tropaeoli* from spontaneous cocoa bean fermentations”. In: *International Journal of Systematic and Evolutionary Microbiology* 63.5, pp. 1709–1716.

Soemphol, W., A. Deeraksa, M. Matsutani, T. Yakushi, H. Toyama, O. Adachi, M. Yamada, and K. Matsushita (2011). “Global analysis of the genes involved in the thermotolerance mechanism of thermotolerant *Acetobacter tropicalis* SKU1100.” In: *Bioscience, biotechnology, and biochemistry* 75.10, pp. 1921–1928.

Sogawa, K., M. Watanabe, K. Sato, S. Segawa, C. Ishii, A. Miyabe, S. Murata, T. Saito, and F. Nomura (2011). “Use of the MALDI

BioTyper system with MALDI-TOF mass spectrometry for rapid identification of microorganisms." In: *Analytical and Bioanalytical Chemistry* 400.7, pp. 1905–1911.

Sokollek, S. J., C. Hertel, and W. P. Hammes (1998). "Description of *Acetobacter oboediens* sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations". In: *International Journal of Systematic and Evolutionary Microbiology* 48.3, pp. 935–940.

Solieri, L. and P. Giudici (2009). "Vinegars of the World". In: *Vinegars of the World*. Milano: Springer Milan, pp. 1–16.

Spitaels, F., A. Wieme, T. Balzarini, I. Cleenwerck, A. Van Landschoot, L. De Vuyst, and P. Vandamme (2014a). "*Gluconobacter cerevisiae* sp. nov., isolated from the brewery environment". In: *International Journal of Systematic and Evolutionary Microbiology* 64.4, pp. 1134–1141.

Spitaels, F., L. Li, A. Wieme, T. Balzarini, I. Cleenwerck, A. Van Landschoot, L. De Vuyst, and P. Vandamme (2014b). "*Acetobacter lambici* sp. nov., isolated from fermenting lambic beer". In: *International Journal of Systematic and Evolutionary Microbiology* 64.4, pp. 1083–1089.

Sreeramulu, G., Y. Zhu, and W. Knol (2000). "Kombucha fermentation and its antimicrobial activity". In: *Journal of Agricultural and Food Chemistry* 48.6, pp. 2589–2594.

Stackebrandt, E., R. G. E. Murray, and H. G. Truper (1988). "Proteobacteria classis nov., a name for the phylogenetic taxon that includes the "purple bacteria and their relatives"". In: *International Journal of Systematic and Evolutionary Microbiology* 38.3, pp. 321–325.

Stackebrandt, E., W. Frederiksen, G. M. Garrity, P. A. D. Grimont, P. Kämpfer, M. C. J. Maiden, X. Nesme, R. Rossello-Mora, J. Swings, H. G. Trüper, L. Vauterin, A. C. Ward, and W. B. WHITMAN (2002). "Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology." In: *International Journal of Systematic and Evolutionary Microbiology* 52.3, pp. 1043–1047.

Stoop, J., J. Williamson, and D. Masonpharr (1996). “Mannitol metabolism in plants: a method for coping with stress”. In: *Trends in Plant Science* 1.5, pp. 139–144.

Strohalm, M., D. Kavan, P. Novák, M. Volný, and V. Havlíček (2010). “mMass 3: a cross-platform software environment for precise analysis of mass spectrometric data”. In: *Analytical Chemistry* 82.11, pp. 4648–4651.

Suzuki, R., Y. Zhang, T. Iino, Y. Kosako, K. Komagata, and T. Uchimura (2010). “*Asaia astilbes* sp. nov., *Asaia platycodi* sp. nov., and *Asaia prunellae* sp. nov., novel acetic acid bacteria isolated from flowers in Japan”. In: *Journal of General and Applied Microbiology* 56.4, pp. 339–346.

Takai, M., Y. Tsuta, and S. Watanabe (1975). “Biosynthesis of cellulose by *Acetobacter xylinum*. I. Characterizations of bacterial cellulose”. In: *Polymer Journal* 7.2, pp. 137–146.

Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar (2013). “MEGA6: Molecular evolutionary genetics analysis version 6.0”. In: *Molecular Biology and Evolution* 30.12, pp. 2725–2729.

Tanasupawat, S., C. Thawai, P. Yukphan, D. Moonmangmee, T. Itoh, O. Adachi, and Y. Yamada (2004). “*Gluconobacter thailandicus* sp nov., an acetic acid bacterium in the *alpha-Proteobacteria*”. In: *Journal of General and Applied Microbiology* 50.3, pp. 159–167.

Tanasupawat, S., J. Kommanee, T. Malimas, P. Yukphan, Y. Nakagawa, and Y. Yamada (2009). “Identification of *Acetobacter*, *Gluconobacter*, and *Asaia* strains isolated in Thailand based on 16S-23S rRNA gene internal transcribed spacer restriction and 16S rRNA gene sequence analyses.” In: *Microbes and Environments* 24.2, pp. 135–143.

Tanasupawat, S., J. Kommanee, P. Yukphan, D. Moonmangmee, Y. Muramatsu, Y. Nakagawa, and Y. Yamada (2011a). “*Gluconobacter uchimurae* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*”. In: *Journal of General and Applied Microbiology* 57.5, pp. 293–301.

Tanasupawat, S., J. Kommanee, P. Yukphan, Y. Muramatsu, Y. Nakagawa, and Y. Yamada (2011b). “*Acetobacter farinalis* sp nov., an

acetic acid bacterium in the *alpha-Proteobacteria*". In: *Journal of General and Applied Microbiology* 57.3, pp. 159–167.

Tanigawa, K., H. Kawabata, and K. Watanabe (2010). "Identification and typing of *Lactococcus lactis* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry". In: *Applied and Environmental Microbiology* 76.12, pp. 4055–4062.

Tazato, N., M. Nishijima, Y. Handa, R. Kigawa, C. Sano, and J. Sugiyama (2012). "*Gluconacetobacter tumulicola* sp. nov. and *Gluconacetobacter asukensis* sp. nov., isolated from the stone chamber interior of the Kitora Tumulus". In: *International Journal of Systematic and Evolutionary Microbiology* 62.8, pp. 2032–2038.

Teoh, A. L., G. Heard, and J. Cox (2004). "Yeast ecology of Kombucha fermentation". In: *International Journal of Food Microbiology* 95.2, pp. 119–126.

Thompson, C. C., G. R. Amaral, M. Campeao, R. A. Edwards, M. F. Polz, B. E. Dutilh, D. W. Ussery, T. Sawabe, J. Swings, and F. L. Thompson (2015). "Microbial taxonomy in the post-genomic era: Rebuilding from scratch?" In: *Archives of Microbiology* 197.3, pp. 359–370.

Thompson, C. C., L. Chimetto, R. A. Edwards, J. Swings, E. Stackebrandt, and F. L. Thompson (2013a). "Microbial genomic taxonomy". In: *BMC Genomics* 14.1, pp. 913–913.

Thompson, C. C., G. G. Z. Silva, N. M. Vieira, R. Edwards, A. C. P. Vicente, and F. L. Thompson (2013b). "Genomic taxonomy of the genus *prochlorococcus*." In: *Microbial Ecology* 66.4, pp. 752–762.

Tindall, B. J. (1988). "Fully saturated menaqionones in the archaebacterium *Pyrobaculum islandicum*". In: *FEMS Microbiology Letters* 60.3, pp. 251–253.

Tindall, B. J., R. Rosselló-Mora, H. J. Busse, W. Ludwig, and P. Kämpfer (2010). "Notes on the characterization of prokaryote strains for taxonomic purposes". In: *International Journal of Systematic and Evolutionary Microbiology* 60.1, pp. 249–266.

Toyosaki, H., Y. Kojima, T. Tsuchida, K. Hoshino, Y. Yamada, and F. Yoshinaga (1995). "The characterization of an acetic acid bacterium useful for producingbacterial cellulose in agitation cultures: The pro-

posal of *Acetobacter xylinum* subsp. *sucrofermentans* subsp. nov." In: *Journal of General and Applied Microbiology* 41.4, pp. 307–314.

Trček, J. and M. Teuber (2002). "Genetic and restriction analysis of the 16S-23S rDNA internal transcribed spacer regions of the acetic acid bacteria". In: *FEMS Microbiology Letters* 208.1, pp. 69–75.

Trček, J. and F. Barja (2014). "Updates on quick identification of acetic acid bacteria with a focus on the 16S—23S rRNA gene internal transcribed spacer and the analysis of cell proteins by MALDI-TOF mass spectrometry". In: *International Journal of Food Microbiology*, pp. 1–39.

Updegraff, D. M. (1969). "Semimicro determination of cellulose in biological materials." In: *Analytical Biochemistry* 32.3, pp. 420–424.

Urakami, T., J. Tamaoka, K. Suzuki, and K. Komagata (1989). "*Acidomonas* gen. nov., incorporating *Acetobacter methanolicus* as *Acidomonas methanolica* comb. nov". In: *International Journal of Systematic Bacteriology* 39.1, pp. 50–55.

Valentine, N., S. Wunschel, D. Wunschel, C. Petersen, and K. Wahl (2005). "Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry." In: *Applied and Environmental Microbiology* 71.1, pp. 58–64.

Van Keer, C., P. V. Abeele, J. Swings, F. Gosselé, and J. De Ley (1981). "Acetic acid bacteria as causal agents of browning and rot of apples and pears". In: *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene: I. Abt. Originale C: Allgemeine, angewandte und ökologische Mikrobiologie* 2.2, pp. 197–204.

Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters, and J. Swings (1996). "Polyphasic taxonomy, a consensus approach to bacterial systematics." In: *Microbiological Reviews* 60.2, pp. 407–438.

Vandamme, P. and C. Peeters (2014). "Time to revisit polyphasic taxonomy." In: *Antonie van Leeuwenhoek* 106.1, pp. 57–65.

Vargha, M., Z. Takáts, A. Konopka, and C. H. Nakatsu (2006). "Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates." In: *Journal of Microbiological Methods* 66.3, pp. 399–409.

Vaz-Moreira, I., M. F. Nobre, O. C. Nunes, and C. M. Manaia (2007). "*Gulbenkiania mobilis* gen. nov., sp. nov., isolated from treated municipal wastewater". In: *International Journal of Systematic and Evolutionary Microbiology* 57.5, pp. 1108–1112.

Veen, S. Q. van, E. C. J. Claas, and E. J. Kuijper (2010). "High-throughput identification of bacteria and yeast by matrix-assisted laser desorption ionization time-of-flight mass spectrometry in conventional medical microbiology laboratories." In: *Journal of Clinical Microbiology* 48.3, pp. 900–907.

Vu, H., P. Yukphan, and W. Chaipitakchonlatarn (2013). "*Nguyenibacter vanlangensis* gen. nov., sp. nov., an unusual acetic acid bacterium in the α-Proteobacteria". In: *Journal of General and Applied Microbiology* 59.3, pp. 239–244.

Wang, D., H. Wang, Y. Zhou, Q. Zhang, F. Zhang, P. Du, S. Wang, C. Chen, and B. Kan (2011). "Genome sequencing reveals unique mutations in characteristic metabolic pathways and the transfer of virulence genes between *V. mimicus* and *V. cholerae*." In: *PloS One* 6.6, e21299.

Watson, J. D. and F. H. Crick (1953a). "Genetical implications of the structure of deoxyribonucleic acid." In: *Nature* 171.4361, pp. 964–967.

Watson, J. D. and F. H. Crick (1953b). "Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid." In: *Nature* 171.4356, pp. 737–738.

Wayne, L. G., D. J. Brenner, R. R. Colwell, P. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, M. Wec, R. Murray, E. Stackebrandt, M. P. Starr, and H. G. Truper (1987). "Report of the Ad-Hoc-Committee on reconciliation of approaches to bacterial systematics". In: *International Journal of Systematic Bacteriology* 37.4, pp. 463–464.

Welker, M. and E. R. B. Moore (2011). "Applications of whole-cell matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry in systematic microbiology." In: *Systematic and Applied Microbiology* 34.1, pp. 2–11.

White, J. W. (1962). *Composition of American Honeys*. Technical bulletin (United States. Department of Agriculture). U.S. Department of Agriculture.

Wieme, A., I. Cleenwerck, A. Van Landschoot, and P. Vandamme (2012). "*Pediococcus lolii* DSM 19927^T and JCM 15055^T are strains of *Pediococcus acidilactici*". In: *International Journal of Systematic and Evolutionary Microbiology* 62.12, pp. 3105–3108.

Wieme, A., F. Spitaels, M. Aerts, K. De Bruyne, A. Van Landschoot, and P. Vandamme (2014a). "Effects of growth medium on matrix-assisted laser desorption-ionization time of flight mass spectra: a case study of acetic acid bacteria." In: *Applied and Environmental Microbiology* 80.4, pp. 1528–1538.

Wieme, A., F. Spitaels, M. Aerts, K. De Bruyne, A. Van Landschoot, and P. Vandamme (2014b). "Identification of beer-spoilage bacteria using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry". In: *International Journal of Food Microbiology* 185.C, pp. 41–50.

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey (1990). "DNA polymorphisms amplified by arbitrary primers are useful as genetic-markers". In: *Nucleic Acids Research* 18.22, pp. 6531–6535.

Williams, T. L., D. Andrzejewski, J. O. Lay, and S. M. Musser (2003). "Experimental factors affecting the quality and reproducibility of MALDI TOF mass spectra obtained from whole bacteria cells". In: *Journal of the American Society for Mass Spectrometry* 14.4, pp. 342–351.

Wu, J. J., Y. K. Ma, F. F. Zhang, and F. S. Chen (2012). "Biodiversity of yeasts, lactic acid bacteria and acetic acid bacteria in the fermentation of "Shanxi aged vinegar", a traditional Chinese vinegar". In: *Food Microbiology* 30.1, pp. 289–297.

Wunschel, D. S., E. A. Hill, J. S. McLean, K. Jarman, Y. A. Gorby, N. Valentine, and K. Wahl (2005). "Effects of varied pH, growth rate and temperature using controlled fermentation and batch culture on matrix assisted laser desorption/ionization whole cell protein fingerprints". In: *Journal of Microbiological Methods* 62.3, pp. 259–271.

Yamada, Y. (2000). "Transfer of *Acetobacter oboediens* Sokollek et al 1998 and *Acetobacter intermedius* Boesch et al. 1998 to the genus

Gluconacetobacter as *Gluconacetobacter oboediens* comb. nov. and *Gluconacetobacter intermedius* comb. nov." In: *International journal of systematic and evolutionary microbiology* 50 Pt 6, pp. 2225–2227.

Yamada, Y. and M. Akita (1984). "An electrophoretic comparison of enzymes in strains of *Gluconobacter* species." In: *The Journal of General and Applied Microbiology*.

Yamada, Y., K. Hoshino, and T. Ishikawa (1997). "The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus Gluconoacetobacter to the generic level." In: *Bioscience, Biotechnology and Biochemistry* 61.8, pp. 1244–1251.

Yamada, Y., N. Itakura, M. Yamashita, and Y. Tahara (1984). "Deoxyribonucleic acid homologies in atrains of *Gluconobacter* species: taxonomic studies on acetic acid bacteria and allied organisms. Part VIII." In: *Journal of Fermentation Technology* 62.6, pp. 595–600.

Yamada, Y., K. Katsura, H. Kawasaki, Y. Widyastuti, S. Saono, T. Seki, T. Uchimura, and K. Komagata (2000). "*Asaia bogorensis* gen. nov., sp nov., an unusual acetic acid bacterium in the alpha-Proteobacteria". In: *International Journal of Systematic and Evolutionary Microbiology* 50, pp. 823–829.

Yamada, Y. and P. Yukphan (2008). "Genera and species in acetic acid bacteria". In: *International Journal of Food Microbiology* 125.1, pp. 15–24.

Yamada, Y., P. Yukphan, H. T. L. Vu, Y. Muramatsu, D. Ochaikul, S. Tanapasuwat, and Y. Nakagawa (2012). "Description of *Komagataeibacter* gen. nov., with proposals of new combinations (*Acetobacteraceae*)". In: *Journal of General and Applied Microbiology* 58.5, pp. 397–404.

Yamashita, S. i. (2004). "Emendation of the genus *Acidomonas* Urakami, Tamaoka, Suzuki and Komagata 1989". In: *International Journal of Systematic and Evolutionary Microbiology* 54.3, pp. 865–870.

Yarza, P., W. Ludwig, J. Euzéby, R. Amann, K.-H. Schleifer, F. O. Glöckner, and R. Rosselló-Móra (2010). "Update of the all-species living tree project based on 16S and 23S rRNA sequence analyses." In: *Systematic and Applied Microbiology* 33.6, pp. 291–299.

Yarza, P., M. Richter, J. Peplies, J. Euzeby, R. Amann, K.-H. Schleifer, W. Ludwig, F. O. Glöckner, and R. Rosselló-Móra (2008). "The all-species living tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains." In: *Systematic and Applied Microbiology* 31.4, pp. 241–250.

Yarza, P., P. Yilmaz, E. Pruesse, F. O. Gloeckner, W. Ludwig, K.-H. Schleifer, W. B. WHITMAN, J. Euzeby, R. Amann, and R. Rossello-Mora (2014). "Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences". In: *Nature Reviews Microbiology* 12.9, pp. 635–645.

Yetiman, A. E. and Z. Kesmen (2015). "Identification of acetic acid bacteria in traditionally produced vinegar and mother of vinegar by using different molecular techniques." In: *International journal of food microbiology* 204, pp. 9–16.

Yukphan, P., T. Malimas, W. Potacharoen, S. Tanasupawat, M. Tanticharoen, and Y. Yamada (2005). "*Neoasaia chiangmaiensis* gen. nov, sp nov., a novel osmotolerant acetic acid bacterium in the *alpha-proteobacteria*". In: *Journal of General and Applied Microbiology* 51.5, pp. 301–311.

Yukphan, P., W. Potacharoen, S. Tanasupawat, M. Tanticharoen, and Y. Yamada (2004a). "*Asaia krungthepensis* sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*". In: *International Journal of Systematic and Evolutionary Microbiology* 54.2, pp. 313–316.

Yukphan, P., M. Takahashi, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2004b). "*Gluconobacter albidus* (ex Kondo and Ameyama 1958) sp. nov., nom. rev., an acetic acid bacterium in the *alpha-Proteobacteria*". In: *Journal of General and Applied Microbiology* 50.4, pp. 235–242.

Yukphan, P., T. Malimas, T. Lundaa, Y. Muramatsu, M. Takahashi, M. Kaneyasu, S. Tanasupawat, Y. Nakagawa, K.-i. Suzuki, M. Tanticharoen, and Y. Yamada (2010). "*Gluconobacter wancherniae* sp. nov., an acetic acid bacterium from Thai isolates in the *alpha-Proteobacteria*". In: *The Journal of General and Applied Microbiology* 56.1, pp. 67–73.

Yukphan, P., T. Malimas, Y. Muramatsu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, M. Tanticharoen, and Y. Yamada (2011). "*Neokomagataea* gen. nov., with descriptions of *Neokomagataea thailandica sp.* nov. and *Neokomagataea tanensis sp.* nov., osmotolerant acetic acid bacteria of the *alpha-Proteobacteria*". In: *Bioscience, Biotechnology and Biochemistry* 75.3, pp. 419–426.

Yukphan, P., T. Malimas, Y. Muramatsu, M. Takahashi, M. Kaneyasu, W. Potacharoen, S. Tanasupawat, Y. Nakagawa, K. Hamana, Y. Tahara, K.-i. Suzuki, M. Tanticharoen, and Y. Yamada (2009). "*Ameyamaea chiangmaiensis* gen. nov., sp. nov., an acetic acid bacterium in the *alpha-Proteobacteria*". In: *Bioscience, Biotechnology and Biochemistry* 73.10, pp. 2156–2162.

Yukphan, P., T. Malimas, Y. Muramatsu, M. Takahashi, M. Kaneyasu, S. Tanasupawat, Y. Nakagawa, K.-i. Suzuki, W. Potacharoen, and Y. Yamada (2008). "*Tanticharoenia sakaeratensis* gen. nov., sp. nov., a new osmotolerant acetic acid bacterium in the *alpha-Proteobacteria*". In: *Bioscience, Biotechnology and Biochemistry* 72.3, pp. 672–676.

Zouache, K., D. Voronin, V. Tran-Van, and P. Mavingui (2009). "Composition of bacterial communities associated with natural and laboratory populations of *Asobara tabida* infected with *Wolbachia*." In: *Applied and Environmental Microbiology* 75.11, pp. 3755–3764.

Part VII.

Annex

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1261	*Acetobacter aceti*		Beech-wood shavings of vinegar plant
LMG 1372	*Acetobacter aceti*		
LMG 1496	*Acetobacter aceti*		
LMG 1504^{T}	*Acetobacter aceti*		Beech-wood shavings of vinegar plant
LMG 1505	*Acetobacter aceti*	Zwolle	Quick vinegar
LMG 1512	*Acetobacter aceti*	Aichi, Japan	Film in fermentor of rice vinegar
LMG 1525	*Acetobacter aceti*	Zwolle, The Netherlands	Quick vinegar
LMG 1535	*Acetobacter aceti*	Belgium	Vinegar plant
LMG 1695	*Acetobacter aceti*		
LMG 18491 t1	*Acetobacter aceti*	Kolinska, Slovenia	Cider vinegar
LMG 18492	*Acetobacter aceti*	Kolinska, Slovenia	Cider vinegar
LMG 1544	*Acetobacter cerevisiae*		Beer
LMG 1545	*Acetobacter cerevisiae*	Aichi, Japan	Film in fermentor of rice vinegar
LMG 1546	*Acetobacter cerevisiae*		
LMG 1549	*Acetobacter cerevisiae*		Top fermentation storage beer
LMG 1585	*Acetobacter cerevisiae*	Ireland	Beer
LMG 1592	*Acetobacter cerevisiae*		Manufacture of vinegar
LMG 1596	*Acetobacter cerevisiae*		
LMG 1599	*Acetobacter cerevisiae*	United Kingdom	Brewers' yeast and beer
LMG 1600	*Acetobacter cerevisiae*	United Kingdom	Brewers' yeast and beer
LMG 1602	*Acetobacter cerevisiae*	United Kingdom	Brewers' yeast and beer
LMG 1608	*Acetobacter cerevisiae*	The Netherlands	Beer
LMG 1625^{T}	*Acetobacter cerevisiae*	Toronto, Canada	Beer (ale) in storage
LMG 1643	*Acetobacter cerevisiae*		Manufacture of vinegar
LMG 1645	*Acetobacter cerevisiae*		
LMG 1647	*Acetobacter cerevisiae*	The Netherlands	Beer
LMG 1652	*Acetobacter cerevisiae*	Ireland	Beer
LMG 1653	*Acetobacter cerevisiae*	Ireland	Beer

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1654	*Acetobacter cerevisiae*	Ireland	Beer
LMG 1655	*Acetobacter cerevisiae*	United Kingdom	Farm cider
LMG 1682	*Acetobacter cerevisiae*	Ireland	Beer
LMG 1684 t1	*Acetobacter cerevisiae*		Beer
LMG 1684 t2	*Acetobacter cerevisiae*		Beer
LMG 1685 t1	*Acetobacter cerevisiae*		
LMG 1705	*Acetobacter cerevisiae*	United Kingdom	Brewery
LMG 21418^T	*Acetobacter cibinongensis*	Indonesia	*Annona montanae*
LMG 27059	*Acetobacter cibinongensis*	Finland	Brewery
LMG 1572	*Acetobacter estunensis*	Bristol, United Kingdom	Cider
LMG 1580	*Acetobacter estunensis*	Leiden, The Netherlands	Beer
LMG 1626^T	*Acetobacter estunensis*	Bristol, United Kingdom	Cider
LMG 1665	*Acetobacter fabarum*		Experimental vinegar production from fermented apple juice
LMG 24244^T	*Acetobacter fabarum*	Ghana	Fermented cocoa beans
LMG 24630	*Acetobacter fabarum*	Gembloux, Belgium	Kefir grains (dairy starter)
LMG 27039	*Acetobacter fabarum*	Central Thailand	Mushroom
LMG 27041	*Acetobacter fabarum*	Central Thailand	Fruit of *Artocarpus heterophyllus*
LMG 27044	*Acetobacter fabarum*	Central Thailand	Flower of *Heliconia* sp.
R-36331	*Acetobacter fabarum*	Ghana	Fermented cocoa beans
R-36458	*Acetobacter fabarum*	Ghana	Fermented cocoa beans
R-36459	*Acetobacter fabarum*	Ghana	Fermented cocoa beans
R-49107	*Acetobacter fabarum*	Brazil	Cocoa bean fermentation
R-49126	*Acetobacter fabarum*	Ecuador	Cocoa bean fermentation
R-49135	*Acetobacter fabarum*	Malaysia	Cocoa bean fermentation

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 26772^T	*Acetobacter farinalis*	Thailand	Fermented starch
LMG 27045	*Acetobacter farinalis*	Central Thailand	Fermented starch
LMG 27046	*Acetobacter farinalis*	Central Thailand	Fermented starch
LMG 23848^T	*Acetobacter ghanensis*	Ghana	Fermented cocoa beans
LMG 27093	*Acetobacter ghanensis*	Pathumthani, Thailand	Peach
R-29336	*Acetobacter ghanensis*	Ghana	Fermented cocoa beans
R-29338	*Acetobacter ghanensis*	Ghana	Fermented cocoa beans
LMG 1571	*Acetobacter indonesiensis*		
LMG 1588	*Acetobacter indonesiensis*		
LMG 1607	*Acetobacter indonesiensis*		Vinegar
LMG 19824^T	*Acetobacter indonesiensis*	Indonesia	Fruit of zirzak (*Annona muricata*)
LMG 27037	*Acetobacter indonesiensis*	Northern Thailand	Fruit of *Aglaia* ap.
LMG 27096	*Acetobacter indonesiensis*	Uttaradit, Thailand	Fermented rice flour
LMG 27439^T	*Acetobacter lambici*	Belgium	Fermenting lambic beer (Industrial type brewery)
LMG 27440	*Acetobacter lambici*	Belgium	Fermenting lambic beer (Traditional type brewery)
R-50193	*Acetobacter lambici*	Belgium	Fermenting lambic beer (Traditional type brewery)
R-50194	*Acetobacter lambici*	Belgium	Fermenting lambic beer (Industrial type brewery)
LMG 1617^T	*Acetobacter lovaniensis*	Becquevoort, Belgium	Sewage on soil
LMG 27097	*Acetobacter lovaniensis*	Uttaradit, Thailand	Fermented rice flour
LMG 1502 t1	*Acetobacter malorum*		
LMG 1552	*Acetobacter malorum*		Probably *Malus* sp.
LMG 1587	*Acetobacter malorum*		Beer (ale)

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1597	*Acetobacter malorum*	United Kingdom	Brewers' yeast and beer
LMG 1598	*Acetobacter malorum*	United Kingdom	Brewers' yeast and beer
LMG 1604	*Acetobacter malorum*	United Kingdom	Sour beer
LMG 1642	*Acetobacter malorum*		
LMG 1646 t1	*Acetobacter malorum*		
LMG 1646 t2	*Acetobacter malorum*		
LMG 1651	*Acetobacter malorum*	United Kingdom	Sample of bottled ale
LMG 1698	*Acetobacter malorum*	United Kingdom	Brewery
LMG 1699	*Acetobacter malorum*	United Kingdom	Brewery
LMG 1700	*Acetobacter malorum*	United Kingdom	Brewery
LMG 1746^{T}	*Acetobacter malorum*	Ghent, Belgium	Rotting apple
R-49137	*Acetobacter malorum*	Malaysia	Cocoa bean fermentation
LMG 23498^{T}	*Acetobacter nitrogenifigens*	India	Kombucha tea
LMG 1586	*Acetobacter oeni*	United Kingdom	Apple juice
LMG 21952^{T}	*Acetobacter oeni*	Viseu Dao region, Portugal	Spoiled red wine
LMG 1803	*Acetobacter okinawensis*	United Kingdom	*Malus sylvestris*
LMG 1804	*Acetobacter okinawensis*	United Kingdom	*Malus sylvestris*
LMG 26457^{T}	*Acetobacter okinawensis*	Okinawa, Japan	Stem of sugarcane
R-49111	*Acetobacter okinawensis*	Ecuador	Cocoa bean fermentation
R-49118	*Acetobacter okinawensis*	Ecuador	Cocoa bean fermentation
R-49119	*Acetobacter okinawensis*	Ecuador	Cocoa bean fermentation
R-49136	*Acetobacter okinawensis*	Malaysia	Cocoa bean fermentation

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1547	*Acetobacter orientalis*	Lockyer Vally Queensland, Australia	Black earth soil
LMG 1576	*Acetobacter orientalis*		
LMG 1664	*Acetobacter orientalis*	United Kingdom	Fermenting putrified meat sample
LMG 1671	*Acetobacter orientalis*	United Kingdom	Fermenting putrified meat sample
LMG 1672	*Acetobacter orientalis*	United Kingdom	Fermenting putrified meat sample
LMG 21417^T	*Acetobacter orientalis*	Indonesia	*Canna hybrida*
LMG 27038	*Acetobacter orientalis*	Central Thailand	Flower of *Canna* sp.
LMG 27042	*Acetobacter orientalis*	Central Thailand	Fruit
LMG 27043	*Acetobacter orientalis*	Central Thailand	Fruit of *Artocarpus heterophyllus*
LMG 27098	*Acetobacter orientalis*	Pathumthani, Thailand	Loog-pang khaomak
LMG 1282	*Acetobacter orleanensis*	Belgium	Beer
LMG 1583^T	*Acetobacter orleanensis*	Belgium	Beer
LMG 27270	*Acetobacter orleanensis*		
LMG 26456^T	*Acetobacter papayae*	Okinawa, Japan	Papaya fruit
LMG 27040	*Acetobacter papayae*	Central Thailand	Fruit of *Psidium guajava*
LMG 1262^T	*Acetobacter pasteurianus*	The Netherlands	Beer
LMG 1497 t1	*Acetobacter pasteurianus*		
LMG 1497 t2	*Acetobacter pasteurianus*		
LMG 1513	*Acetobacter pasteurianus*	Aichi, Japan	Film in fermentor of rice vinegar
LMG 1514	*Acetobacter pasteurianus*	Aichi, Japan	Film in fermentor of rice vinegar
LMG 1540	*Acetobacter pasteurianus*	Japan	
LMG 1541	*Acetobacter pasteurianus*	Japan	
LMG 1543	*Acetobacter pasteurianus*	East Africa	Vinegar brews
LMG 1548	*Acetobacter pasteurianus*		
LMG 1550	*Acetobacter pasteurianus*		
LMG 1551	*Acetobacter pasteurianus*		
LMG 1553	*Acetobacter pasteurianus*	Osaka, Japan	Spoiled beer
LMG 1554	*Acetobacter pasteurianus*		

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1555	*Acetobacter pasteurianus*		
LMG 1556	*Acetobacter pasteurianus*		
LMG 1577	*Acetobacter pasteurianus*		
LMG 1590	*Acetobacter pasteurianus*		
LMG 1591	*Acetobacter pasteurianus*		Quick vinegar
LMG 1594	*Acetobacter pasteurianus*		
LMG 1603	*Acetobacter pasteurianus*		Vinegar brews
LMG 1605	*Acetobacter pasteurianus*	East Africa	Vinegar brews
LMG 1606	*Acetobacter pasteurianus*	The Netherlands	Beer
LMG 1609	*Acetobacter pasteurianus*		
LMG 1610	*Acetobacter pasteurianus*		
LMG 1611	*Acetobacter pasteurianus*		
LMG 1612	*Acetobacter pasteurianus*		
LMG 1613	*Acetobacter pasteurianus*	The Netherlands	Beer
LMG 1615 t1	*Acetobacter pasteurianus*	The Netherlands	Beer
LMG 1616	*Acetobacter pasteurianus*	The Netherlands	Beer
LMG 1618	*Acetobacter pasteurianus*	United Kingdom	Malt vinegar acetifier
LMG 1620	*Acetobacter pasteurianus*		Malt vinegar brewery acetifiers
LMG 1622	*Acetobacter pasteurianus*		Malt vinegar brewery acetifiers
LMG 1623	*Acetobacter pasteurianus*		
LMG 1624 t1	*Acetobacter pasteurianus*		
LMG 1629	*Acetobacter pasteurianus*	Recife, Brazil	Fermented *Agave sisalana* juice
LMG 1630	*Acetobacter pasteurianus*	Recife, Brazil	*Saccharum officinarum* bagasse
LMG 1631	*Acetobacter pasteurianus*	Recife, Brazil	Acidified *Saccharum officinarum* juice
LMG 1632	*Acetobacter pasteurianus*	Africa	African vinegar
LMG 1638	*Acetobacter pasteurianus*		
LMG 1639	*Acetobacter pasteurianus*	The Netherlands	Beer
LMG 1640	*Acetobacter pasteurianus*		
LMG 1649 t1	*Acetobacter pasteurianus*		
LMG 1649 t2	*Acetobacter pasteurianus*		
LMG 1650	*Acetobacter pasteurianus*		
LMG 1658	*Acetobacter pasteurianus*	Myanmar	
LMG 1659	*Acetobacter pasteurianus*	Myanmar	

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1666	*Acetobacter pasteurianus*		Fermented alcoholic mash
LMG 1685 t2	*Acetobacter pasteurianus*		
LMG 1686	*Acetobacter pasteurianus*	The Netherlands	Vinegar from dry raisins
LMG 23182	*Acetobacter pasteurianus*	Australia	Oxidized Shiraz wine showing spoilage
LMG 26310	*Acetobacter pasteurianus*	Switzerland	Red wine vinegar (submerged methodology)
LMG 27050	*Acetobacter pasteurianus*	Thailand	Fruit
LMG 27051	*Acetobacter pasteurianus*	Thailand	Red wine
LMG 6039	*Acetobacter pasteurianus*		
R-29340	*Acetobacter pasteurianus*	Ghana	Fermented cocoa beans
R-29341	*Acetobacter pasteurianus*	Ghana	Fermented cocoa beans
R-29342	*Acetobacter pasteurianus*	Ghana	Fermented cocoa beans
LMG 1633	*Acetobacter peroxydans*	Netherlands	Ditch water
LMG 1635^T	*Acetobacter peroxydans*	Delft, The Netherlands	Ditch water
LMG 21769	*Acetobacter peroxydans*	Tamilnadu, India	Root tissue of *Oryza sativa*
LMG 21770	*Acetobacter peroxydans*	Tamilnadu, India	Root tissue of wetland rice
R-49133	*Acetobacter peroxydans*	Malaysia	Cocoa bean fermentation
R-50323	*Acetobacter peroxydans*	Malaysia	Cocoa bean fermentation
R-50324	*Acetobacter peroxydans*	Malaysia	Cocoa bean fermentation
R-50325	*Acetobacter peroxydans*	Malaysia	Cocoa bean fermentation
LMG 1805	*Acetobacter persici*	United Kingdom	*Malus sylvestris*
LMG 26458^T	*Acetobacter persici*	Tottori Prefecture, Japan	A peach fruit
LMG 27271	*Acetobacter persici*		
R-49105	*Acetobacter persici*	Brazil	Cocoa bean box fermentation
R-49106	*Acetobacter persici*	Brazil	Cocoa bean fermentation

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
R-49108	*Acetobacter persici*	Brazil	Cocoa bean fermentation
R-49109	*Acetobacter persici*	Brazil	Cocoa bean fermentation
R-49110	*Acetobacter persici*	Brazil	Cocoa bean fermentation
R-49112	*Acetobacter persici*	Ecuador	Cocoa bean fermentation
R-49113	*Acetobacter persici*	Ecuador	Cocoa bean fermentation
R-49116	*Acetobacter persici*	Ecuador	Cocoa bean fermentation
R-49127	*Acetobacter persici*	Ecuador	Cocoa bean fermentation
R-49130	*Acetobacter persici*	Ecuador	Cocoa bean fermentation
R-49131	*Acetobacter persici*	Malaysia	Cocoa bean fermentation
LMG 18848^{T}	*Acetobacter pomorum*	Esslingen, Germany	Cider vinegar fermentation
LMG 23690^{T}	*Acetobacter senegalensis*	Casamance, Senegal	Mango fruit
LMG 27036	*Acetobacter senegalensis*	Thailand	
LMG 27049	*Acetobacter senegalensis*	Thailand	
LMG 27052	*Acetobacter senegalensis*	Northern Thailand	Fruit of *Annona squamosa*
LMG 27053	*Acetobacter senegalensis*	Thailand	Rose apple
R-29339	*Acetobacter senegalensis*	Ghana	Fermented cocoa beans
R-29343	*Acetobacter senegalensis*	Ghana	Fermented cocoa beans
LMG 1501	*Acetobacter sicerae*		
LMG 1508	*Acetobacter sicerae*		
LMG 1530	*Acetobacter sicerae*		Cider
LMG 1531^{T}	*Acetobacter sicerae*		Celluloseless mutant of LMG 1530 isolated from cider
LMG 1670	*Acetobacter sicerae*	United Kingdom	Fermenting putrified meat sample
LMG 27543	*Acetobacter sicerae*	Belgium	Kefir
LMG 28092	*Acetobacter sicerae*	Belgium	Kefir
LMG 5	*Acetobacter sicerae*	Belgium	Beer

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1627	*Acetobacter* sp. nov. I		
LMG 1637	*Acetobacter* sp. nov. II		
LMG 1636	*Acetobacter* sp. nov. II		Starch producing mutant of an Acetobacter pasteurianus strain
LMG 1506	*Acetobacter* sp. nov. III	Belgium	Beer
LMG 1706	*Acetobacter* sp. nov. III	United Kingdom	Brewery
LMG 21419[T]	*Acetobacter syzygii*	Indonesia	*Syzyguim malaccense*
LMG 27101	*Acetobacter syzygii*	Bangkok, Thailand	Sapodilla
LMG 1663	*Acetobacter tropicalis*	United Kingdom	Fermenting putrified meat sample
LMG 1754	*Acetobacter tropicalis*	Ivory Coast	Fruit of Ficus capensis
LMG 19825[T]	*Acetobacter tropicalis*	Indonesia	Coconut (*Coccos nucifera*)
LMG 19826	*Acetobacter tropicalis*	Indonesia	Lime
R-49132	*Acetobacter tropicalis*	Malaysia	Cocoa bean fermentation
LMG 1667	*Acidomonas methanolica*		Sludge
LMG 1668[T]	*Acidomonas methanolica*	Leipzig, Germany	Fermentation of methanol with *Candida* sp. (not sterile)
LMG 1669	*Acidomonas methanolica*		Sludge
LMG 1735	*Acidomonas methanolica*	Australia	Surface microflora of *Saccharum officinarum*
LMG 27010[T]	*Ameyamaea chiangmaiensis*	Chaing Mai, Thailand	Flower of red ginger (*Alpinia purpurea*)
NBRC 103197	*Ameyamaea chiangmaiensis*	Chaing Mai, Thailand	Flower of red ginger (*Alpinia purpurea*)
LMG 1426	*Asaia astilbes*	Aalter, Belgium	*Solidago canadensis*
LMG 26974[T]	*Asaia astilbes*	Japan, Yamanashi Prefecture	*Astilbe thunbergii var.congesta*

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 27005	*Asaia astilbes*	Niigata, Japan	Manyspiny knotweed (*Persicaria senticosa*)
LMG 27006	*Asaia astilbes*	Niigata, Japan	Asian dayflower (*Commelina communis*)
LMG 28297	*Asaia astilbes*	Belgium	Gut of a bumble bee
LMG 21650^T	*Asaia bogorensis*	Bogor, Indonesia	Flower of orchid tree (*Bauhinia purpurea*)
LMG 23141	*Asaia bogorensis*	Bogor, Indonesia	Flower of orchid tree (*Bauhinia purpurea*)
LMG 23142	*Asaia bogorensis*	Bogor, Indonesia	Flower of *Plumbago auriculata*
LMG 23143	*Asaia bogorensis*	Yogyakarta, Indonesia	Tape kefan (fermented glutinous rice)
LMG 27034	*Asaia bogorensis*	Central Thailand	Flower of *Citharexylum spinosum*
LMG 27285	*Asaia bogorensis*	Chiang Mai, Thailand	Flower of *Allamanda cathatica*
NBRC 103412	*Asaia bogorensis*	Bangkok Thailand	Flower of *Hibiscus* sp.
NBRC 103479	*Asaia bogorensis*	Thong Pha Phum,Thailand	Flower of *Ipomoea* sp.
LMG 23083^T	*Asaia krungthepensis*	Bangkok, Thailand	Flower of *Heliconia* sp.
LMG 23139	*Asaia krungthepensis*	Bangkok, Thailand	Flower of *Heliconia* sp.
LMG 23140	*Asaia krungthepensis*	Bangkok, Thailand	Flower of *Heliconia* sp.
LMG 27030	*Asaia krungthepensis*	Central Thailand	Flower of *Heliconia* sp.
LMG 27033	*Asaia krungthepensis*	Central Thailand	Flower of *Heliconia* sp.
LMG 27283	*Asaia krungthepensis*	Nonthaburi, Thailand	Fruit of *Coccinia grandis*
NBRC 103419	*Asaia krungthepensis*	Bang Bua Tong, Thailand	Fruit of *Sandoricum indicum*
LMG 27011^T	*Asaia lannensis*	Chiang Mai, Thailand	Flower of spider lily (*Crinum asiaticum*)
NBRC 102527	*Asaia lannensis*	Chiang Mai, Thailand	Fower of spider lily (*Crinum asiaticum*)

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1760	*Asaia platycodi*	The Netherlands	Apple soft drink
LMG 1762	*Asaia platycodi*	The Netherlands	Orange stilldrink
LMG 26975^{T}	*Asaia platycodi*	Japan, Akita	Balloon flower of *Platycodon grandiflorum*
LMG 27002	*Asaia platycodi*	Tokyo, Japan	Unidentified flower
LMG 26976^{T}	*Asaia prunellae*	Japan, Akita	Self-heal (*Prunella vulgaris*)
LMG 27003	*Asaia prunellae*	Akita, Japan	Self-heal (*Prunella vulgaris*)
LMG 27004	*Asaia prunellae*	Akita, Japan	Unidentified flower
LMG 21651^{T}	*Asaia siamensis*	Bangkok, Thailand	Crown flower (*Calotropis gigantea*)
LMG 23136	*Asaia siamensis*	Bogor, Indonesia	Flower of spider lily (*Crinum asiaticum*)
LMG 23144	*Asaia siamensis*	Bogor, Indonesia	Flower of spider lily (*Crinum asiaticum*)
LMG 23145	*Asaia siamensis*	Bogor, Indonesia	Flower of spider lily (*Crinum asiaticum*)
LMG 23146	*Asaia siamensis*	Bogor, Indonesia	Flower of *Ixora chinensis*
LMG 27284	*Asaia siamensis*	Northern Thailand	Flower of *Ixora* sp.
LMG 1453	*Asaia* sp. nov.		
LMG 27047^{T}	*Asaia spathodeae*	Thailand	Flower of *Spathodea companulata*
LMG 27288	*Asaia spathodeae*	Thailand	Flower of *Spathodea companulata*
NBRC 103415	*Asaia spathodeae*	Samutsakorn, Thailand	Fruit of pomelo (*Citrus maxima*)
LMG 28161T	*Bombella intestini*	Belgium	Crop of a bumble bee
R-52486	*Bombella intestini*	Belgium	Crop of a bumble bee
LMG 1352	*Bombella* sp. nov.	Madrid area, Spain	Beehive
LMG 1354	*Bombella*sp. nov.	Madrid area, Spain	Beehive
LMG 27436^{T}	*“Commensalibacter intestini”*		Gut of *Drosophila melanogaster*

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 28296	*"Commensalibacter intestini"*	Belgium	Gut of a bumble bee
LMG 26838^{T}	*Endobacter medicaginis*	Zamora, Spain	Surface-sterilized nodule of *Medicago sativa*
LMG 1752	*Enterococcus* sp.		
LMG 27801^{T}	*Gluconacetobacter aggeris*	Asuka village, Japan	Soil
LMG 27724^{T}	*Gluconacetobacter asukensis*	Asuka village, Japan	A brown viscous gel in the stone chamber of the Kitora Tumulus
LMG 21311^{T}	*Gluconacetobacter azotocaptans*	Tapachula Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23156	*Gluconacetobacter azotocaptans*	Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23157	*Gluconacetobacter azotocaptans*	Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 1732	*Gluconacetobacter diazotrophicus*	Australia	Surface microflora of *Saccharum officinarum*
LMG 1733	*Gluconacetobacter diazotrophicus*	Australia	Saccharum officinarum, surface microflora
LMG 1734	*Gluconacetobacter diazotrophicus*	Australia	*Saccharum officinarum*, surface microflora
LMG 1736 t1	*Gluconacetobacter diazotrophicus*	Australia	*Saccharum officinarum*, surface microflora
LMG 22174	*Gluconacetobacter diazotrophicus*	Tamilnadu, India	Root tissue of *Oryza sativa*
LMG 25582	*Gluconacetobacter diazotrophicus*	Bois Rouge, Mauritius	Sugercane trash
LMG 25583	*Gluconacetobacter diazotrophicus*	Sodnac Quatre Bornes, Mauritius	Leaf sheath of sugercane
LMG 7602	*Gluconacetobacter diazotrophicus*	Alagoas, Brazil	*Saccharum officinarum*, root

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 7603^T	*Gluconacetobacter diazotrophicus*	Alagoas, Brazil	Root of *Saccharum officinarum*
LMG 7971	*Gluconacetobacter diazotrophicus*	Pernambuco, Brazil	Stem of *Saccharum officinarum*
LMG 8065	*Gluconacetobacter diazotrophicus*	Rio de Janeiro, Brazil	Stem of Saccharum officinarum
LMG 8066	*Gluconacetobacter diazotrophicus*	Alagoas, Brazil	*Saccharum officinarum*, root
LMG 8067	*Gluconacetobacter diazotrophicus*	Rio de Janeiro, Brazil	Root of *Saccharum officinarum*
LMG 8068	*Gluconacetobacter diazotrophicus*	Rio de Janeiro, Brazil	*Saccharum officinarum*, rhizosphere
R-40435	*Gluconacetobacter diazotrophicus*	Mauritius	Sugarcane fields
LMG 21312^T	*Gluconacetobacter johannae*	Tapachula Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23153	*Gluconacetobacter johannae*	Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 23154	*Gluconacetobacter johannae*	Motozintla Chiapas, Mexico	Rhizosphere of *Coffea arabica L.*
LMG 1347	*Gluconacetobacter liquefaciens*	Japan	Fruit
LMG 1348	*Gluconacetobacter liquefaciens*	Japan	Fruit
LMG 1381^T	*Gluconacetobacter liquefaciens*	Japan	Dried fruit of *Diospyros* sp.
LMG 1388	*Gluconacetobacter liquefaciens*	Japan	Fruit
LMG 1503	*Gluconacetobacter liquefaciens*		
LMG 1509	*Gluconacetobacter liquefaciens*		Canal water
LMG 1532	*Gluconacetobacter liquefaciens*	Japan	Fruit
LMG 27031	*Gluconacetobacter liquefaciens*	Central Thailand	Flower of *Heliconia* sp.
LMG 27032	*Gluconacetobacter liquefaciens*	Central Thailand	*Helicona* sp.
LMG 27035	*Gluconacetobacter liquefaciens*	Northern Thailand	Fruit of *Manikara zapota*
LMG 1736 t2	*Gluconacetobacter sacchari*	Australia	*Saccharum officinarum*, surface microflora
LMG 19747^T	*Gluconacetobacter sacchari*	Queensland, Australia	Leaf sheath of sugar cane

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 19748	*Gluconacetobacter sacchari*	Queensland, Australia	Mealy bug
LMG 25587	*Gluconacetobacter sacchari*	Moka, Mauritius	Sugercane trash
LMG 1728	*Gluconacetobacter takamatsuzukensis*	Hawaii, United States	*Ananas comosus*, pink diseased fruit
LMG 27800^{T}	*Gluconacetobacter takamatsuzukensis*	Japan	Soil in Takamatsuzuka Tumulus
LMG 27725^{T}	*Gluconacetobacter tumulicola*	Asuka village, Japan	A black viscous substance in the stone chamber of the Kitora Tumulus
LMG 27802^{T}	*Gluconacetobacter tumulisoli*	Asuka village, Japan	Clay soil in Takamatsuzuka Tumulus
LMG 1356^{T}	*Gluconobacter albidus*	Japan	*Dahlia coccinea*
LMG 1375	*Gluconobacter albidus*	Nishinomiya Japan	Fruit of *Myrica rubra*
LMG 1409	*Gluconobacter albidus*		Cider
LMG 1431 t1	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1431 t2	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1432	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1434	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1440	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1442	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1443	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1450	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1455	*Gluconobacter albidus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1559	*Gluconobacter albidus*	Japan	*Lilium auratum*
LMG 1768	*Gluconobacter albidus*	The Netherlands	Orange lemonade
LMG 1350	*Gluconobacter cerevisiae*		

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1351	*Gluconobacter cerevisiae*		
LMG 1430	*Gluconobacter cerevisiae*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1704	*Gluconobacter cerevisiae*	United Kingdom	Brewery
LMG 1707	*Gluconobacter cerevisiae*	United Kingdom	Brewery
LMG 27748^T	*Gluconobacter cerevisiae*	Belgium	Fermenting lambic beer
LMG 27749	*Gluconobacter cerevisiae*	Belgium	Spoiled brewer's yeast starter culture
LMG 27882	*Gluconobacter cerevisiae*	Belgium	A traditional lambic brewery
LMG 1362	*Gluconobacter cerinus*	Japan	*Malus* sp.
LMG 1368^T	*Gluconobacter cerinus*	Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1376 t1	*Gluconobacter cerinus*	Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1376 t2	*Gluconobacter cerinus*	Nishinomiya, Japan	*Myrica rubra*, fruit
LMG 1389	*Gluconobacter cerinus*	Herbal garden, Kyoto, Japan	Flower of *Rheum rhabarbarum*
LMG 1390	*Gluconobacter cerinus*	Herbal garden, Kyoto, Japan	Flower of *Rheum rhabarbarum*
LMG 1416	*Gluconobacter cerinus*		
LMG 1420	*Gluconobacter cerinus*		
LMG 1425	*Gluconobacter cerinus*		
LMG 1427	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1428	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1429	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1433	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1435	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1436	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1437	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1438	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1439	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1441	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1444	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1445	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1446	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1447 t1	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1447 t2	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1448	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1449	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1451	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1452	*Gluconobacter cerinus*	Aalter, Belgium	*Helenium* sp.
LMG 1454	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1456	*Gluconobacter cerinus*	Aalter, Belgium	Honeybee (*Apis mellifera*)
LMG 1457	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
LMG 1458	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
LMG 1459	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
LMG 1460	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
LMG 1461	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
LMG 1462	*Gluconobacter cerinus*	Neeroeteren, Belgium	Honeybee (*Apis mellifera*)
LMG 1463	*Gluconobacter cerinus*	near Zottegem, Belgium	Honeybee (*Apis mellifera*)
LMG 1464	*Gluconobacter cerinus*	Zottegem, Belgium	Honeybee (*Apis mellifera*)
LMG 1465	*Gluconobacter cerinus*	Zottegem, Belgium	Honeybee (*Apis mellifera*)
LMG 1475	*Gluconobacter cerinus*	Zottegem, Belgium	Honeybee (*Apis mellifera*)

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1679 t1	*Gluconobacter cerinus*	Leiden, The Netherlands	Beer
LMG 1679 t2	*Gluconobacter cerinus*	Leiden, The Netherlands	Beer
LMG 1718	*Gluconobacter cerinus*	Alberton, South Africa	Lager beer at fermentation stage
LMG 1747	*Gluconobacter cerinus*	Belgium	*Malus* sp.
LMG 1749	*Gluconobacter cerinus*	Ghent, Belgium	Rotting apple
LMG 1763	*Gluconobacter cerinus*	The Netherlands	Orange lemonade
LMG 1766	*Gluconobacter cerinus*	The Netherlands	Raspberry stilldrink
LMG 1767	*Gluconobacter cerinus*	The Netherlands	Black current lemonade
LMG 1357 t2	*Gluconobacter frateurii*	Botanical garden, Kyoto, Japan	Flower of *Dahlia* sp.
LMG 1358	*Gluconobacter frateurii*	Botanical garden, Kyoto, Japan	Flower of *Dahlia* sp.
LMG 1363	*Gluconobacter frateurii*	Osaka, Japan	*Fragaria ananassa*
LMG 1365^T	*Gluconobacter frateurii*	Osaka, Japan	*Fragaria ananassa*
LMG 1366	*Gluconobacter frateurii*	Osaka, Japan	*Fragaria ananassa*
LMG 1369 t1	*Gluconobacter frateurii*	Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1369 t2	*Gluconobacter frateurii*	Osaka, Japan	*Prunus* sp. (cherry)
LMG 1371	*Gluconobacter frateurii*	Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1378	*Gluconobacter frateurii*	Osaka, Japan	*Eriobotrya japonica*, fruit
LMG 1379	*Gluconobacter frateurii*	Osaka, Japan	Fruit of *Eriobotrya japonica*
LMG 1419	*Gluconobacter frateurii*		
LMG 1421	*Gluconobacter frateurii*		
LMG 1492	*Gluconobacter frateurii*	Osaka, Japan	*Diospyros* sp.
LMG 1570	*Gluconobacter frateurii*	Osaka, Japan	*Fragaria ananassa*
LMG 1656	*Gluconobacter frateurii*		
LMG 1737	*Gluconobacter frateurii*	Ghent, Belgium	*Rotting pear*
LMG 1738	*Gluconobacter frateurii*	Ghent, Belgium	*Pyrus communis*
LMG 1281	*Gluconobacter japonicus*	Nishinomiya, Japan	*Myrica rubra*, fruit
LMG 1364	*Gluconobacter japonicus*	Osaka, Japan	*Fragaria ananassa*

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1370	*Gluconobacter japonicus*	Osaka, Japan	Cherry (*Prunus* sp.)
LMG 1373^T	*Gluconobacter japonicus*	Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1374	*Gluconobacter japonicus*	Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1383	*Gluconobacter japonicus*	Japan	Fruit
LMG 1397	*Gluconobacter japonicus*		
LMG 1407	*Gluconobacter japonicus*	Leuven, Belgium	Bakers' yeast
LMG 1417	*Gluconobacter japonicus*		
LMG 1574	*Gluconobacter japonicus*		
LMG 1575	*Gluconobacter japonicus*	Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1675	*Gluconobacter japonicus*	Leuven, Belgium	Bakers' yeast
LMG 1678 t1	*Gluconobacter japonicus*	Delft, The Netherlands	Beer
LMG 1678 t2	*Gluconobacter japonicus*	Delft, The Netherlands	Beer
LMG 26773	*Gluconobacter japonicus*	Thailand	Rambutan (*Nephelium lappaceum*)
LMG 27103	*Gluconobacter japonicus*	Thailand	Rambutan (*Nephelium lappaceum*)
NBRC 103476	*Gluconobacter japonicus*	Sakaerat, Thailand	Flower
NBRC 103491	*Gluconobacter japonicus*	Tong Pha Phum, Thailand	Fruit
R-49114	*Gluconobacter japonicus*	Ecuador	Cocoa bean fermentation
R-49115	*Gluconobacter japonicus*	Ecuador	Cocoa bean fermentation
R-49117	*Gluconobacter japonicus*	Ecuador	Cocoa bean fermentation
R-49123	*Gluconobacter japonicus*	Ecuador	Cocoa bean fermentation
LMG 26774^T	*Gluconobacter kanchanaburiensis*	Tong Pha Phum, Thailand	Fermented fruit of *Artocarpus heterophyllus*
NBRC 103588	*Gluconobacter kanchanaburiensis*	Tong Pha Phum, Thailand	Fermented fruit of *Artocarpus heterophyllus*

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1367 t1^T	*Gluconobacter kondonii*	Osaka, Japan	*Fragaria ananassa*
NBRC 103510	*Gluconobacter kondonii*	Tong Pha Phum, Thailand	Flower of *Mucuna pruriens*
LMG 27435^T	*"Gluconobacter morbifer"*		Gut of *Drosophila melanogaster*
LMG 1355	*Gluconobacter oxydans*	Japan	*Dahlia coccinea*
LMG 1359	*Gluconobacter oxydans*		
LMG 1384	*Gluconobacter oxydans*		
LMG 1385	*Gluconobacter oxydans*	Osaka, Japan	Dried fruit of *Diospyros* sp.
LMG 1386	*Gluconobacter oxydans*	Osaka, Japan	*Diospyros* sp., dried fruit
LMG 1393	*Gluconobacter oxydans*		
LMG 1394	*Gluconobacter oxydans*		
LMG 1395	*Gluconobacter oxydans*	Botanical garden, Kyoto, Japan	Flower of *Liatris scariosa*
LMG 1398	*Gluconobacter oxydans*	United Kingdom	Beer
LMG 1399	*Gluconobacter oxydans*		Beer
LMG 1400	*Gluconobacter oxydans*		
LMG 1401	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
LMG 1402	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
LMG 1403	*Gluconobacter oxydans*		Derived from ATCC 621
LMG 1404	*Gluconobacter oxydans*		
LMG 1405	*Gluconobacter oxydans*		
LMG 1406	*Gluconobacter oxydans*		Ropy beer
LMG 1408^T	*Gluconobacter oxydans*		Beer
LMG 1410	*Gluconobacter oxydans*		
LMG 1411	*Gluconobacter oxydans*		
LMG 1413	*Gluconobacter oxydans*		
LMG 1422	*Gluconobacter oxydans*		
LMG 1423	*Gluconobacter oxydans*		
LMG 1424	*Gluconobacter oxydans*		
LMG 1484	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
LMG 1485	*Gluconobacter oxydans*		
LMG 1494	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1516	*Gluconobacter oxydans*		
LMG 1519	*Gluconobacter oxydans*	United Kingdom	Ropy sample of top fermented beer
LMG 1533	*Gluconobacter oxydans*		
LMG 1569	*Gluconobacter oxydans*	United Kingdom	Beer
LMG 1581	*Gluconobacter oxydans*	Delft, The Netherlands	Beer
LMG 1589^T	*Gluconobacter oxydans*		Beer
LMG 1673	*Gluconobacter oxydans*	Delft, The Netherlands	Amstel beer
LMG 1674	*Gluconobacter oxydans*	Delft, The Netherlands	Beer
LMG 1676	*Gluconobacter oxydans*	Leuven, Belgium	Beer
LMG 1677	*Gluconobacter oxydans*	Delft, The Netherlands	Beer
LMG 1680	*Gluconobacter oxydans*	South Africa	Sour porridge
LMG 1681	*Gluconobacter oxydans*		derived from ATCC 621
LMG 1683	*Gluconobacter oxydans*		Irish beer
LMG 1691	*Gluconobacter oxydans*		
LMG 1764	*Gluconobacter oxydans*	The Netherlands	Cider lemonade
LMG 1765	*Gluconobacter oxydans*	The Netherlands	Lemon lemonade
LMG 1785	*Gluconobacter oxydans*	Ireland	Beer
LMG 27012	*Gluconobacter oxydans*	Thailand	Rakam fruit (*Zalacca wallichiana*)
LMG 27013	*Gluconobacter oxydans*	Thailand	Litchi fruit
LMG 27014	*Gluconobacter oxydans*	Bangkok, Thailand	Jujube fruit
LMG 27015	*Gluconobacter oxydans*	Bangkok, Thailand	Longan fruit
LMG 27054	*Gluconobacter oxydans*	Thailand	
R-49120	*Gluconobacter oxydans*	Ecuador	Cocoa bean fermentation
R-49121	*Gluconobacter oxydans*	Ecuador	Cocoa bean fermentation
LMG 1412	*Gluconobacter roseus*	Tokyo, Japan	*Diospyros* sp., fruit
LMG 1418^T	*Gluconobacter roseus*	Tokyo, Japan	Fruit of *Diospyros* sp.
LMG 1745	*Gluconobacter* sp.	Ghent, Belgium	Rotting apple

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1414^{T}	*Gluconobacter sphaericus*	Japan	Grapes
LMG 1744	*Gluconobacter sphaericus*	Ghent, Belgium	Rotting pear
NBRC 103509	*Gluconobacter sphaericus*	Tong Pha Phum, Thailand	Fruit of apple guava (*Psidium guajava*)
NBRC 103521	*Gluconobacter sphaericus*	Tong Pha Phum, Thailand	Fruit
LMG 1377	*Gluconobacter thailandicus*		
LMG 1486	*Gluconobacter thailandicus*	Osaka, Japan	*Fragaria ananassa*
LMG 1487	*Gluconobacter thailandicus*	Osaka, Japan	*Fragaria ananassa*
LMG 1488	*Gluconobacter thailandicus*	Osaka, Japan	Fruit of *Prunus tomentosa*
LMG 1489	*Gluconobacter thailandicus*	Osaka, Japan	*Prunus tomentosa*, fruit
LMG 1490	*Gluconobacter thailandicus*	Osaka, Japan	*Prunus tomentosa*, fruit
LMG 1491	*Gluconobacter thailandicus*	Osaka, Japan	*Diospyros* sp.
LMG 1493	*Gluconobacter thailandicus*	Osaka, Japan	*Diospyros* sp.
LMG 1739	*Gluconobacter thailandicus*	Ghent, Belgium	*Pyrus communis*
LMG 1740	*Gluconobacter thailandicus*	Ghent, Belgium	*Pyrus communis*
LMG 1741	*Gluconobacter thailandicus*	Ghent, Belgium	Rotting pear
LMG 1742	*Gluconobacter thailandicus*	Ghent, Belgium	*Pyrus communis*
LMG 1743	*Gluconobacter thailandicus*	Ghent, Belgium	Rotting pear
LMG 23137^{T}	*Gluconobacter thailandicus*	Bangkok, Thailand	Flower of the Indian cork tree (*Millingtonia hortensis*)
LMG 23138	*Gluconobacter thailandicus*	Bangkok, Thailand	Flower of glossy ixora (*Ixora lobbii*)
LMG 27016^{T}	*Gluconobacter wancherniae*	Nakhon Ratchasima, Thailand	Seed
NBRC 103582	*Gluconobacter wancherniae*	Nakhon Ratchasima, Thailand	Fruit

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 24392^T	*Granulibacter bethesdensis*	Bethesda, United States	Patient with chronic granulomatous disease, lymph node
LMG 12	*Komagataeibacter europaeus*		
LMG 1380	*Komagataeibacter europaeus*	Nishinomiya, Japan	Fruit of *Myrica rubra*
LMG 1510	*Komagataeibacter europaeus*	Copenhagen, Denmark	Vinegar
LMG 1520	*Komagataeibacter europaeus*	Kenya	Vinegar brews
LMG 1521	*Komagataeibacter europaeus*	Nairobi, Kenya	Vinegar brew
LMG 1523	*Komagataeibacter europaeus*	East Africa	Vinegar
LMG 1641	*Komagataeibacter europaeus*		African vinegar brew
LMG 1690	*Komagataeibacter europaeus*		
LMG 1696	*Komagataeibacter europaeus*	Nishinomiya, Japan	*Myrica rubra*, fruit
LMG 18494	*Komagataeibacter europaeus*	Ljubljana Slovenia	Red wine vinegar produced in submerged bioreactor
LMG 18890^T	*Komagataeibacter europaeus*	Esslingen, Germany	Submerged culture vinegar generator
LMG 20956	*Komagataeibacter europaeus*	Ljubljana, Slovenia	Cider vinegar produced in industrial submerged bioreactor
LMG 26311	*Komagataeibacter europaeus*	Switzerland	Red wine vinegar (submerged methodology)
LMG 1524	*Komagataeibacter hansenii*	Jerusalem, Israel	Vinegar
LMG 1527^T	*Komagataeibacter hansenii*		Celluloseless mutant 1 from NCIB 8745 from vinegar
LMG 1528	*Komagataeibacter hansenii*		Celluloseless mutant 2 from NCIB 8745 from vinegar
LMG 23726	*Komagataeibacter hansenii*	India	Kombucha tea ferment
LMG 27060	*Komagataeibacter hansenii*		
LMG 27286	*Komagataeibacter hansenii*	Northern Thailand	Beleric myrobalan
LMG 18909^T	*Komagataeibacter intermedius*	Switzerland	Commercially available kombucha

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 26206^T	*Komagataeibacter kakiaceti*	Kumamoto Prefecture, Japan	Kaki vinegar
LMG 27001	*Komagataeibacter kakiaceti*	Kumamoto Prefecture, Japan	Kaki vinegar
LMG 1529^T	*Komagataeibacter maltaceti*	United Kingdom	Malt vinegar brewery acetifiers
LMG 1526	*Komagataeibacter medellinensis*		Beer
LMG 1692	*Komagataeibacter medellinensis*		
LMG 1693^T	*Komagataeibacter medellinensis*	Japan	Vinegar
LMG 1536T	*Komagataeibacter nataicola*	Manila, Philippines	Nata de coco
LMG 1517	*Komagataeibacter oboediens*		
LMG 1522	*Komagataeibacter oboediens*		
LMG 1688	*Komagataeibacter oboediens*		
LMG 1689	*Komagataeibacter oboediens*		
LMG 18849^T	*Komagataeibacter oboediens*	Esslingen, Germany	Red wine vinegar fermentation
LMG 18907	*Komagataeibacter oboediens*	Ljubljana, Slovenia	Cider vinegar produced in submerged bioreactor
LMG 18908	*Komagataeibacter oboediens*	Ljubljana, Slovenia	Cider vinegar produced in submerged bioreactor
LMG 26312	*Komagataeibacter oboediens*	Switzerland	Spirit vinegar (submerged methodology)
LMG 22126^T	*Komagataeibacter rhaeticus*	Val Venosta South Tyrol, Italy	Organic apple juice
LMG 27048	*Komagataeibacter rhaeticus*	Central Thailand	Rambutan
LMG 27414	*Komagataeibacter rhaeticus*		
LMG 27415	*Komagataeibacter rhaeticus*		
LMG 1502 t2	*Komagataeibacter saccharivorans*		
LMG 1582^T	*Komagataeibacter saccharivorans*	Germany	Beet juice
LMG 1584	*Komagataeibacter saccharivorans*		
LMG 18788^T	*Komagataeibacter sucrofermentans*	Tokyo, Japan	Black cherry

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 27287	*Komagataeibacter sucrofermentans*	Central Thailand	Fetid passinflower
LMG 25	*Komagataeibacter swingsii*		
LMG 22125^{T}	*Komagataeibacter swingsii*	Val Venosta South Tyrol, Italy	Organic apple juice
LMG 1515^{T}	*Komagataeibacter xylinus*		Mountain ash berries
LMG 1518	*Komagataeibacter xylinus*	Verona	
LMG 1538	*Komagataeibacter xylinus*		
LMG 21812^{T}	*Kozakia baliensis*	Bali, Indonesia	Palm brown sugar
LMG 27017	*Kozakia baliensis*	Yogyakarta, Indonesia	Ragi (starter)
LMG 27018	*Kozakia baliensis*	Yogyakarta, Indonesia	Ragi (starter)
LMG 27019	*Kozakia baliensis*	Yogyakarta, Indonesia	Ragi (starter)
LMG 24037^{T}	*Neoasaia chiangmaiensis*	Chiang-Mai, Thailand	Flower of red ginger (*Alpinia purpurata*)
LMG 27020^{T}	*Neokomagataea tanensis*	Thailand	Flower of candle bush (*Senna alata*)
LMG 27021^{T}	*Neokomagataea thailandica*	Thailand	Flower of spanish flag (*Lantana camera*)
LMG 1353	*Pantoea* sp.	Near Madrid, Spain	Beehive
LMG 1757	*Rosenbergiella collisarenosi*		
LMG 23170^{T}	*Saccharibacter floricola*	Kanagawa Prefecture, Japan	Pollen
LMG 21291^{T}	*Swaminathania salitolerans*	India	*Porteresia coarctata*
LMG 27022^{T}	*Tanticharoenia sakaeratensis*	Thailand	Soil
NBRC 103194	*Tanticharoenia sakaeratensis*	Thailand	Soil
NBRC 103195	*Tanticharoenia sakaeratensis*	Thailand	Soil
LMG 1573	*Tatumella* sp.		
LMG 1723	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
LMG 1725	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit

List of strains and isolates investigated in the present study.

Strain	Species	Geographic origin	Biological origin
LMG 1729	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
LMG 1770	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
LMG 1771	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
LMG 1773	*Tatumella* sp.	Hawaii, United States	*Ananas comosus*, pink diseased fruit
R-49122	*Tatumella* sp.	Ecuador	Cocoa bean fermentation
R-49124	*Tatumella* sp.	Ecuador	Cocoa bean fermentation
R-49129	*Tatumella* sp.	Ecuador	Cocoa bean fermentation