Microbiology of Vinegar: from Isolation, Phenetic Characterization and Detection of Acetic Acid Bacteria to Microbial Profiling of an Industrial Production

João Nuno Serôdio de Melo

Thesis to obtain the Master of Science Degree in

Microbiology

Supervisor(s): Prof. Rogério Paulo de Andrade Tenreiro

Prof. Nuno Gonçalo Pereira Mira

Examination Committee:

Chairperson: Prof. Jorge Humberto Gomes Leitão

Supervisor: Prof. Rogério Paulo de Andrade Tenreiro

Member of the Committee: Prof. Rodrigo da Silva Costa

December, 2016

Acknowledgements

I would like to express my sincere gratitude to my supervisor, Professor Rogério Tenreiro, for giving me this opportunity and for all his support and guidance throughout the last year. I have truly learned immensely working with you.

Also, I would like to show appreciation to my supervisor from IST, Professor Nuno Mira, for his support along the development of this thesis.

I would also like to thank Professor Ana Tenreiro for all her time and help, especially with the flow cytometry assays. Additionally, I'd like to thank Professor Lélia Chambel for her help with the Bionumerics software.

I would like to thank Filipa Antunes, from the Lab Bugworkers | M&B-BioISI, for the remarkable organization of the lab and for all her help.

I would like to express my gratitude to Mendes Gonçalves, S.A. for the opportunity to carry out this thesis and for providing support during the whole course of this project. Special thanks to Cristiano Roussado for always being available.

Also, a big thank you to my colleagues and friends, Catarina, Sofia, Tatiana, Ana, Joana, Cláudia, Mariana, Inês and Pedro for their friendship, support and for this enjoyable year, especially to Ana Marta Lourenço for her enthusiastic Gram stainings.

Finally, I'd like to thank my family and friends for all the help and support, especially my Dad.

Abstract

Acetic acid bacteria (AAB) are strictly aerobic *Alphaproteobacteria* known for their ability to oxidize ethanol into acetic acid. Currently, one of the limitations of the vinegar industry is the lack of adequate monitoring methodologies, the use of an undefined microbial community as inoculum and the unavailability of starter cultures. AAB were isolated from a variety of vinegars and were grouped into five strains based on two genomic fingerprinting techniques, GTG_5-REP-PCR and PH-RAPD-PCR. Isolates belonging to all strains were selected for the amplification and sequencing of the 16S rRNA gene. Homology search and phylogenetic reconstruction positioned all strains in the genus *Komagataeibacter*, although the identification to the species level of any of the strains was not possible. Four strains were grown in red wine in order to evaluate the suitability of the employment of these strains as starter cultures. Strains 1 and 3 show desirable characteristics of an optimal acetic acid bacteria starter, such as a short lag phase, high cell yield, no ethanol overoxidation and no cellulose production. A molecular detection method for acetic acid bacteria was developed targeting the *adhA* gene. This methodology proved to be fast and reliable in the distinction of acetic acid bacteria from non-AAB isolates. Lastly, microbial community DNA was extracted from five vinegar samples, corresponding to different stages of a red wine vinegar production cycle. Two regions of the 16S rRNA gene were amplified, sequenced by Next Generation Sequencing and identified by homology search. The results showed that *Komagataeibacter* spp. clearly dominate this process.

Keywords: acetic acid bacteria (AAB); genomic fingerprinting; starter culture; *adhA* detection; microbial profiling of vinegar.

Resumo

Bactérias do ácido acético (AAB) são *Alphaproteobacteria* estritamente aeróbicas, famosas pela sua capacidade de oxidação de etanol em ácido acético. Atualmente, algumas das limitações da indústria do vinagre são a inexistência de técnicas de monitorização, o uso de comunidades microbianas indefinidas como inóculo e a não existência de culturas *starter*. AAB foram isoladas de uma variedade de vinagres e agrupadas em cinco estirpes com base em duas metodologias de *fingerprinting* genómico, GTG_5-REP-PCR e PH-RAPD-PCR. Isolados pertencentes a todas as estirpes foram selecionados para amplificação e sequenciação do gene rRNA 16S. Uma análise de homologia e uma reconstrução filogenética posicionou todas as estirpes no género *Komagataeibacter*, apesar da identificação ao nível de espécie não ter sido conseguida. Quatro estirpes foram crescidas em vinho tinto de forma a avaliar a sua aplicabilidade como culturas *starter*. As estirpes 1 e 3 mostraram características desejáveis de uma cultura *starter* de AAB tais como, curta fase de latência, elevado crescimento celular e inexistência de oxidação de ácido acético e de produção de celulose. Um método de detecção molecular com o gene *adhA* como alvo foi desenvolvido. Esta metodologia provou-se ser rápida e fidedigna na distinção entre AAB e isolados não-AAB. Por fim, foi extraído DNA da comunidade microbiana de cinco amostras de vinagre correspondentes a diferentes fases de um ciclo de produção de vinagre de vinho tinto. Duas regiões do gene rRNA 16S foram amplificadas, sequenciadas por NGS e identificadas por uma análise de homologia. Os resultados mostraram que *Komagataeibacter* spp. claramente dominam este processo.

Palavras-chave: bactérias do ácido acético; *fingerprinting* genómico; culturas *starter*; detecção molecular de *adhA*; *microbial profiling* de vinagre.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AAB	acetic acid bacteria
ACS	acetyl-CoA synthase
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
BLAST	Basic Local Alignment Search Tool
CECT	Spanish Type Culture Collection
CIO	cyanide-insensitive oxidase
DGGE	Denaturing Gradient Gel Electrophoresis
DSMZ	German Collection of Microorganisms and Cell Cultures
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus PCR
FAD	flavin adenine dinucleotide
ITS	Internal Transcribed Spacer
MCD	molybdenum-molybdopterin cytosine dinucleotide
MG	Mendes Gonçalves S.A.
MLSA	Multilocus Sequence Analysis
NAD	nicotinamide adenine dinucleotide
NGS	next-generation sequencing
OD	optical density
PQQ	pyrroloquinoline quinone
RAPD-PCR	Randomly Amplified Polymorphic DNA PCR
REP-PCR	Repetitive Extragenic Palindromic PCR
TBV	traditional balsamic vinegar
TCA	tricarboxylic acid
UQ	ubiquinone
VBNC	viable but not cultivable

1. Introduction

1.1 Acetic Acid Bacteria: an Overview

Acetic acid bacteria are Gram negative, rod-shaped, peritrichously or polarly flagellated when motile, mesophilic and obligate aerobes. Most are catalase positive and oxidase negative. These bacteria are capable of oxidizing sugars, sugar alcohols and alcohols to corresponding acids [Komagata *et al*., 2014]. They also exhibit resistance to high acetic acid concentrations at low pH. Acetic acid bacteria not only play a positive role in the production of a variety of foods and beverages, such as vinegars, *kombucha*, cocoa and *nata de coco*, but they can also occur as spoilers of other foods and beverages, such as wine, soft drinks and fruits [Raspor *et al*., 2008]. In recent years, acetic acid bacteria have been the object of extensive research, resulting in a significant restructuration of their taxonomy and advances in understanding their physiology, metabolism and molecular biology and in methods for their isolation and identification [Raspor *et al*., 2008].

The famous ability of this group of bacteria to oxidize ethanol to acetic acid is due to two key membrane-bound enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Both these enzymes are bound to the cytoplasmatic membrane and face the periplasmic space [Sievers & Swings, 2005]. This special type of metabolism differentiates them from all other bacteria. Interestingly, although some of the biotechnological applications of acetic acid bacteria have been practiced for many years, at an industrial scale, much of the knowledge required for a thorough understanding of these processes is still absent. This lack of knowledge is the result of an acknowledged difficulty in handling acetic acid bacteria in a series of routine microbiology techniques, mainly in their isolation and cultivation on solid media [Raspor *et al*., 2008].

1.1.1 Taxonomy

The generic name *Acetobacter* was first introduced for acetic acid bacteria by Beijerinck (1898), with the type species *Acetobacter aceti*. Two other species were subsequently described, *Acetobacter pasteurianus* and *Acetobacter peroxydans* (Beijerinck and Folpmers 1916; Visser't Hooft 1925, respectively). *Gluconobacter* was proposed as a new genus of acetic acid bacteria by Asai (1934, 1935) for strains that lacked the capacity for the oxidation of acetic acid, in contrast to the strains of the genus *Acetobacter*, which were capable of oxidizing acetic acid to carbon dioxide and water [Raspor *et al*., 2008].

In the Approved List of Bacterial Names (Skerman et al. 1980), the family *Acetobacteraceae* was introduced for the two genera of acetic acid bacteria, *Acetobacter* with the three species mentioned above and *Gluconobacter* as a monotypic genus, with *Gluconobacter oxydans* as its type species [Raspor *et al*., 2008]. Since then, the *Acetobacteraceae* family has suffered countless taxonomic changes, where many genera and species have been newly described [Sievers & Swings, 2005].

This family is taxonomically included in the phylum *Proteobacteria*, the class *Alphaproteobacteria* and the order *Rhodospirillales*, and 36 genera are currently described, with standing in nomenclature. *Acetobacter* is the type genus. The genera can be divided in 2 groups, an acetous group and an

acidophilic group, based on phylogeny, ecology and biotechnological applications [Komagata *et al*., 2014]. The acetic acid bacteria are included in the former group, the acetous group. Currently, acetic acid bacteria are distributed in 18 genera, with standing in nomenclature. A list of all genera where acetic acid bacteria are included is presented in Table 1.

Table 1. List of all genera, with standing in nomenclature, where acetic acid bacteria are included, as well as the abbreviation used in this study. The information shown in this table was collected from Matsushita *et al*. (2016), with the exception of the information regarding the genus *Bombella*, which was collected from Li *et al*. (2015).

Genus	Abbreviation	Reference
Acetobacter	*A.*	Beijerinck 1898
Gluconobacter	*G.*	Asai 1935
Acidomonas	*Ac.*	Urakami et al. 1989 emend. Yamashita et al. 2004
Gluconacetobacter	*Ga.*	Yamada et al. 1998
Asaia	*As.*	Yamada et al. 2000
Kozakia	*K.*	Lisdiyanti et al. 2002
Swaminathania	*S.*	Loganathan and Nair 2004
Saccharibacter	*Sc.*	Jojima et al. 2004
Neoasaia	*N.*	Yukphan et al. 2006
Granulibacter	*Gr.*	Greenberg et al. 2006
Tanticharoenia	*T.*	Yukphan et al. 2008
Ameyamaea	*Am.*	Yukphan et al. 2010
Neokomagataea	*Nk.*	Yukphan et al. 2011
Komagataeibacter	*Km.*	Yamada et al. 2013
Endobacter	*E.*	Ramírez-Bahena et al. 2013
Nguyenibacter	*Ng.*	Vu et al. 2013
Swingsia	*Sw.*	Malimas et al. 2014
Bombella	*B.*	Li et al. 2015

Five genera retain most species of acetic acid bacteria: *Acetobacter* (25 species), *Gluconobacter* (14), *Gluconacetobacter* (11), *Asaia* (8) and *Komagataeibacter* (13). The remaining 13 genera are monotypic, with the exception of the genus *Neokomagataea*, which has 2 species. Two genera, *Acetobacter* and *Komagataeibacter*, are generally responsible for the wine vinegar production process.

Identification. Originally, the taxonomy of acetic acid bacteria was based on morphological and physiological criteria. However, phenotypic identification of strains of this group of bacteria, particularly on the species level, is not only inaccurate, but also time consuming. The main reason for this difficulty is the instability of physiological traits, due to the presence of insertion elements in the genome of

acetic acid bacteria, as well as the difficulty in managing these bacteria in routine laboratory techniques, due to their slow growth [Raspor *et al*., 2008].

Recent advances in molecular techniques, as well as the development of new culture media and isolation procedures, have led to the noticeable restructuration of acetic acid bacteria taxonomy and classification. Nowadays, classification of acetic acid bacteria is particularly dependent on molecular approaches. The most common molecular techniques applied revolve around the sequencing or restriction analysis of the 16S rRNA gene and/or of the 16S-23S rRNA Internal Transcribed Spacer (ITS), as well as the application of these molecular targets in Denaturing Gradient Gel Electrophoresis (DGGE). However, the 16S rRNA gene sequences of acetic acid bacteria are very similar to each other, which may cause problems in identification when working solely with this gene [Guillamón *et al*., 2009].

From a biotechnological point of view, the discrimination or typing of different strains can be significant since strains within a species may not exhibit the same phenotypic characteristics, mainly the productivity in terms of ethanol oxidation. Some studies have reported the applicability of molecular techniques to this goal. Generally, PCR-based techniques are employed, such as Randomly Amplified Polymorphic DNA (RAPD-PCR) [Chambel *et al*., 2007; Nanda *et al*., 2001], Repetitive Extragenic Palindromic sequences (REP-PCR) [Cleenwerck *et al*., 2010; De Vuyst *et al*., 2008] and Enterobacterial Repetitive Intergenic Consensus sequences (ERIC-PCR) [Férnandez-Pérez *et al*., 2010; Vegas *et al*., 2010].

Phylogenetic relationships amongst acetic acid bacteria.

The relationships currently acknowledged amongst acetic acid bacteria are represented in Figure 1, extracted from Matsushita *et al*. (2016) with the exception of the genus *Bombella*. Nevertheless, the approximate phylogenetic position of this genus is indicated, based on the information provided by the study describing this new genus [Li *et al*., 2015]. *Acidocella facilis* ATCC 35904^{T}, a member of the acidophilic group of the *Acetobacteraceae* family, was used as an outgroup.

The major taxonomic rearrangements that occurred in the acetous group of this family of bacteria are clearly depicted in the presented phylogeny, namely the elevation to the genus level of the sub-genus *Gluconacetobacter* and of *Acetobacter methanolica*, with the type species *Gluconacetobacter liquefaciens* and *Acidomonas methanolica*, respectively, and the separation of the two subclusters of the genus *Gluconacetobacter* with the proposal of *Komagataeibacter* as a new genus, being *Komagataeibacter xylinus* the type species. The type strain of *Gluconacetobacter entanii* was not available in any culture collection and so, the species could not be listed as a new combination, according to Rule 27 of the Bacteriological Code [Yamada *et al*., 2012]. It is interesting to note that most species of the genera *Gluconacetobacter*, *Komagataeibacter* and *Acidomonas* once belonged to the genus *Acetobacter*.

Since *Acetobacter* and *Komagataeibacter*, as already referred, are mostly implicated in wine vinegar production, a short taxonomic description of each genus is presented.

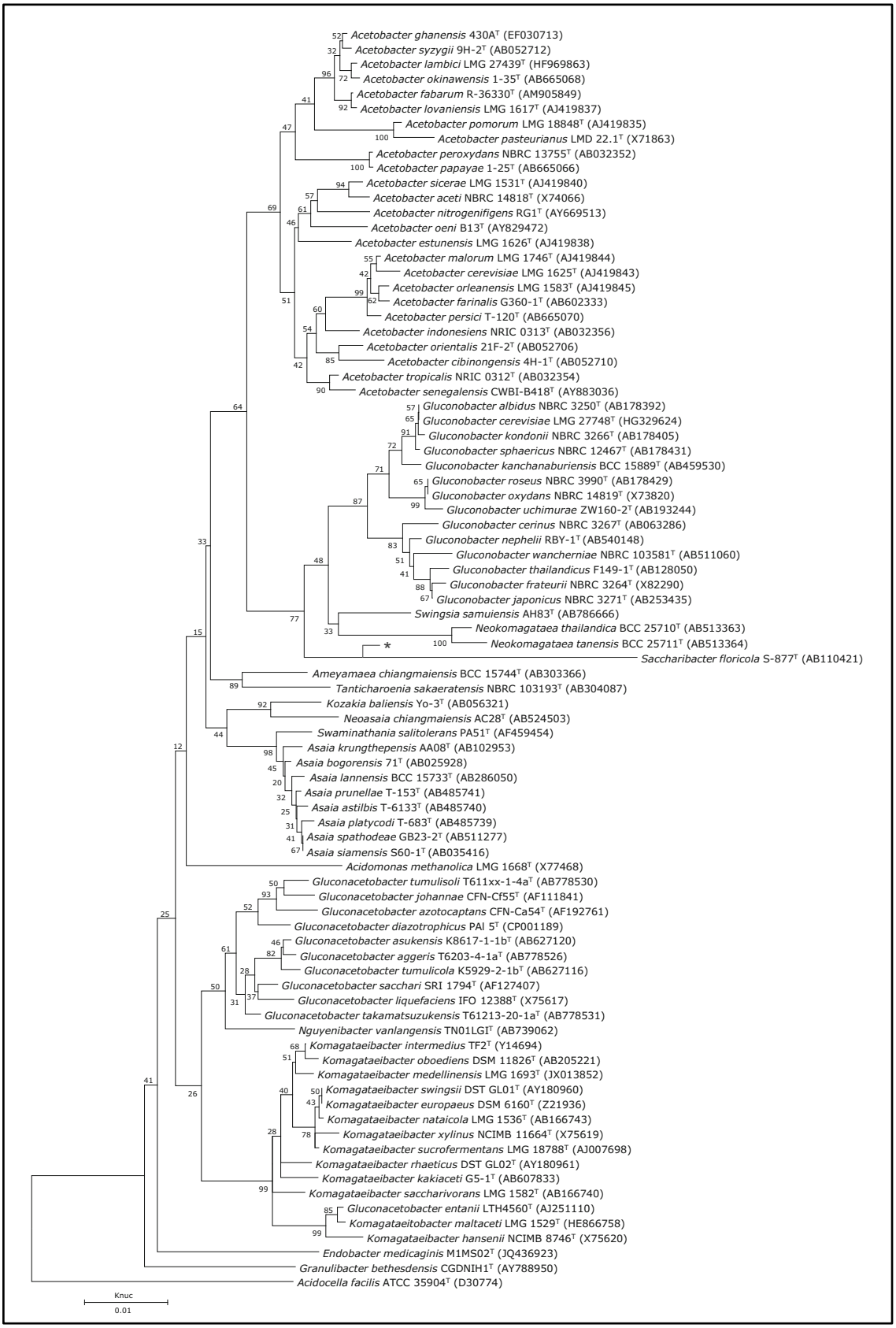


Figure 1. Neighbor-joining phylogenetic tree of acetic acid bacteria. The phylogenetic tree was constructed with Mega 5.05, based on 16S rRNA gene sequences (1213 bases). Numbers at branch nodes represent bootstrap values (%) derived from 1000 replications. *Acidocella facilis* ATCC 35904^T was used as an outgroup. Extracted from Matsushita *et al*. (2016). The (*) indicates the approximate position of the genus *Bombella*, according to Li *et al*. (2015).

Acetobacter Beijerinck 1898

This genus was named after the Latin word for vinegar, *acetum*. *Acetobacter* is the oldest genus in the classification of acetic acid bacteria and is the type genus of the family *Acetobacteraceae*.

Cells are Gram negative rods, ellipsoidal to rod shaped, occurring singly, in pairs or in chains. Most are peritrichously flagellated when motile. Colonies are generally circular, smooth, entire, cream to beige color and opaque. Strictly aerobic, catalase positive (except for *A. peroxydans*) and oxidase negative. A minority of strains produce a water-soluble brown pigment. Acetic acid is produced from ethanol and acetate and lactate are oxidized to carbon dioxide and water. In the type strain of *A. aceti*, acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, D-mannose, or ethanol. Most species are not able to grow on 30% D-glucose (w/v). Optimal growth temperature is around 30ºC. Growth generally occurs at 37ºC and pH 3.5. The major quinone is Q-9. The mol% G+C of the DNA ranges from 53.5 to 60.7 [Matsushita *et al*., 2016, Sievers & Swings, 2005].

Komagataeibacter Yamada et al. 2013

This genus was named in honor of Dr. Kazuo Komagata, a Japanese microbiologist, Professor of The University of Tokyo, Japan, who contributed to bacterial systematics, particularly of acetic acid bacteria. The genus *Gluconacetobacter* was divided into two genera, the genus *Gluconacetobacter* Yamada et al. 1998 and the genus *Komagataeibacter* Yamada et al. 2013, on the basis of 16S rRNA gene and morphological, physiological and ecological characterizations. Ten species of the genus *Gluconacetobacter* were transferred to the genus *Komagataeibacter* as new combinations, with *Komagataeibacter xylinus* as the type species. Recently, three new combinations were described, also transferred from the genus *Gluconacetobacter*, on the basis of phylogenetic relationships [Matsushita *et al*., 2016].

Cells of this genus are Gram negative rods, occurring singly, in pairs or in chains. Most are non-motile. Colonies are described as circular, smooth or rough and white-creamy to beige. Strictly aerobic, catalase positive and oxidase negative. Acetic acid is produced from ethanol and acetate and lactate are oxidized to carbon dioxide and water. Acid is produced from L-arabinose, D-xylose, D-glucose, D-galactose, and ethanol. Cellulosic materials are produced by some strains, particularly of *Km. xylinus* and *Km. nataicola*. A water-soluble brown pigment is not produced on GYC medium. Growth generally occurs in the presence of 0.35% acetic acid (v/v), as well as at pH 3.0. Some species require acetic acid for growth. The major quinone is Q-10. The mol% G+C of the DNA ranges from 58 to 64 [Komagata *et al*., 2014; Matsushita *et al*., 2016].

1.1.2 Ecology

Acetic acid bacteria have been isolated from both natural and artificial environments. Naturally they occur in fruits, flowers, palm sap, garden soil, and various insects, like honeybees, mosquitoes, flies and leafhoppers [Komagata *et al*., 2014; Raspor *et al*., 2008]. Some species have been described as

nitrogen fixers and can be found inhabiting the roots and stems of plants [Raspor *et al*., 2008]. Acetic acid bacteria are also able to grow in artificial and man-made environments, that include vinegar, grape wine, palm wine, soft drinks, cider, beer, kefir, sugar cane juice and canal water [Raspor *et al*., 2008].

Acetobacter strains prefer alcohol-enriched and acidic environments like vinegar, wine, beer and other alcoholic beverages, in contrast to *Gluconobacter* strains, which prefer sugar-enriched environments like fruits and flowers. The genus *Komagataeibacter* has niches in acetous materials such as a variety of vinegars and fruit juices. *Gluconacetobacter* strains have been isolated from vinegar, fruits, dried fruits and the rhizosphere of plants. *Asaia* strains are commonly found in a large variety of flowers. *Acidomonas methanolica* strains were amply isolated from activated sludge samples, but were not found in vegetables or fruits. Strains of the genera *Ameyamaea*, *Neoasaia*, *Neokomagataea*, *Swingsia* and *Bombella* were isolated from a variety of flowers. *Swaminathania* and *Nguyenibacter* were isolated from the rhizosphere of rice. *Kozakia* strains were isolated from palm brown sugar and *Endobacter* strains were isolated from alfalfa. *Granulibacter* strains were isolated from three patients with chronic granulomatous disease. Lastly, a *Tanticharoenia sakaeratensis* strain was isolated from a soil sample and strains from *Saccharibacter floricola* were isolated from pollens in Japan [Komagata *et al*., 2014; Li *et al*., 2015; Matsushita *et al*., 2016].

Pathogenicity. Due to the ecology and the common sources of isolation of acetic acid bacteria, the pathogenicity of this group of bacteria has been entirely ignored until recently. Acetic acid bacteria are clearly different from generally known bacteria of clinical importance when it comes to their growth responses, general phenotype and phylogenetic relationships. Additionally, acetic acid bacteria have shown resistance to a wide variety of antibiotics and, for this reason, have drawn attention from clinicians as newly emerging opportunistic pathogens [Komagata *et al*., 2014].

Almost all of the medical records related to acetic acid bacteria report the isolation of a Gram negative rod unidentifiable by conventional commercial identification systems. These organisms were isolated either from lesions of patients with Chronic Granulomatous Disease, sputum of patients with Cystic Fibrosis or blood of patients with a history of drug use and/or patients undergoing hemo-dialysis for end-stage renal failure. In all cases, identification was only possible by sequencing of the 16S rRNA gene, resulting in identification either to the genus-level or species-level. These isolates include *Gr. bethesdensis*, *As. bogorensis*, *As. lannensis*, *A. cibinogensis*, *A. indonesiensis*, *Ac. methanolica* and *Gluconobacter* spp.. The sources and routes of infection of acetic acid bacteria are still unknown [Komagata *et al*., 2014].

1.1.3 Physiology

As mentioned before, acetic acid bacteria are biochemically quite unique since they are specialized in the incomplete oxidation of sugars, sugar alcohols and ethanol that leads to an uncommon growth behavior and response to extreme culture conditions [Deppenmeier *et al*., 2002]. Because these

oxidative bacteria do not oxidize sugars or alcohols completely to CO_2 and H_2O, or at least not in their early growth phase, they accumulate the corresponding incomplete oxidation products in the growth medium, in large quantities [Matsushita, 2004]. Interestingly, the complete genome sequence of a *Gluconobacter oxydans* strain revealed 75 open reading frames for putative dehydrogenases/oxidoreductases of unknown function [Prust *et al*., 2005].

Gluconobacter species display highly active oxidation reactions on sugars and sugar alcohols, while *Acetobacter* and *Komagataeibacter* species have a highly active ethanol-oxidizing activity but fewer sugar- and sugar alcohol-oxidizing activity [Matsushita, 2004].

In addition to energy generation for cell growth, the oxidative respiration of acetic acid bacteria is critical in the accumulation of oxidative products in their environment. Most of these acidic oxidation products, such as acetic acid, are detrimental to other microorganisms and thus contribute to the fitness of acetic acid bacteria in highly competitive environments, such as fruits and flowers. Furthermore, the rapid oxidation of sugars and sugar alcohols in the media leads to the depletion of available carbon sources, further disrupting competitive microorganisms. Thus, acetic acid bacteria seem to be well adapted to specific environments where high concentration of sugars and sugar alcohols occur, in highly aerobic conditions [Matsushita *et al*., 2016; Prust *et al*., 2005].

Bioenergetics. The respiratory chain of acetic acid bacteria is rather simple with respect to their arrangements of the respiratory components [Matsushita, 2004]. Oxidation reactions of sugars, sugar alcohols and alcohols are essentially carried out by specific membrane-bound dehydrogenases, directly linked to the respiratory chain, anchored in the periplasmic side of the cytoplasmic membrane of the bacteria [Komagata *et al*., 2014]. These membrane-bound dehydrogenases have been classified as quinoproteins and quinoproteins-cytochrome complex (having pyrroloquinoline quinone, PQQ, as a covalently-bound prosthetic group), flavoproteins-cytochrome complex (having flavin adenine dinucleotide, FAD, as a covalently-bound prosthetic group) and molybdoprotein-cytochrome complex (having molybdenum-molybdopterin cytosine dinucleotide, MCD, as a covalently-bound prosthetic group), as illustrated in Figure 2. These organisms lack a proton-translocating NADH:ubiquinone oxidoreductase (complex I) and a cytochrome *c* oxidase (complex IV), which means that they have a limited ability to translocate protons in the course of redox reactions [Prust *et al*., 2005].

The core system is composed of many primary membrane-bound dehydrogenases and terminal ubiquinol oxidase(s), both connected by a ubiquinone (UQ). The UQ present in the respiratory chain of *Acetobacter* species is mainly ubiquinone-9, whereas ubiquinone-10 is mostly present in the remaining genera of acetic acid bacteria [Matsushita, 2004]. Generally, acetic acid bacteria seem to express at least two different types of terminal ubiquinol oxidases, ba_3 (*G. oxydans* seems to express bo_3 type) and cyanide-insensitive oxidase (CIO). The ba_3 type is described as having a proton translocating ability, while CIO type does not. The CIO seems to be more expressed in lower pH conditions, although its physiological function is not yet clear [Matsushita *et al*., 2016; Prust *et al*., 2005]. Oxygen is the final electron acceptor, resulting in the formation of H_2O and proton

motive force necessary for the energy production through a membrane-bound ATP synthase [Mamlouk *et al.*, 2013].

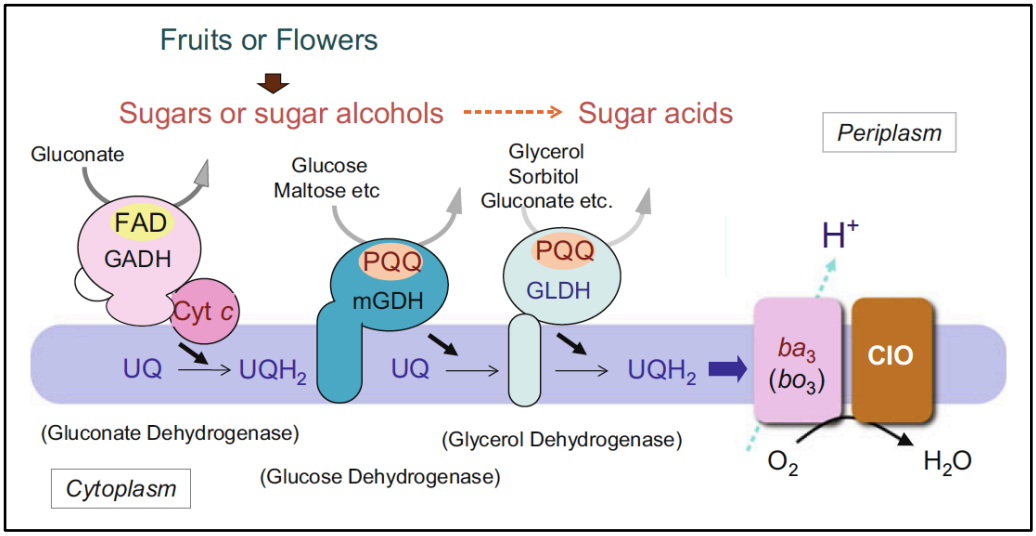


Figure 2. Simplistic representation of the respiratory chain of acetic acid bacteria. It consists of different types of periplasmic dehydrogenases, capable of oxidizing several substrates, that transfer an electron to ubiquinone (UQ), converting it into ubiquinol (UQH_2), and at least two different terminal ubiquinol oxidases, ba_3 (with H^+ pumping ability) and cyanide-insensitive oxidase (CIO), all of which are bound tightly to the cytoplasmic membrane. Extracted from Matsushita *et al.* (2016).

Acetic acid bacteria have developed such a unique respiratory chain that enables them to generate less energy, but a higher electron transfer ability. This adaptation allows them to perform an extensive amount of incomplete oxidations, leading to the accumulation of the resulting products in their environment [Matsushita *et al.*, 2016].

Growth on ethanol. Ethanol oxidation is a process unique to acetic acid bacteria and occurs in two consecutive catalytic reactions performed by two key enzymes, ADH, which is an ethanol:ubiquinone oxidoreductase PQQ dependent and ALDH, which is an aldehyde:ubiquinone oxidoreductase, thought to be MCD dependent. Both these enzymes are bound to the periplasmic side of the cytoplasmic membrane, directly linked to the respiratory chain [Mamlouk *et al.*, 2013]. These enzymes are responsible for the accumulation of acetic acid in the growth medium.

Such unique periplasmic respirations are only involved in the partial oxidation of substrates, but not in their complete oxidation. Assimilation (the complete oxidation) of ethanol occurs at the cytoplasmic level, although both these oxidations (ethanol and acetate) rarely occur at the same time. Ethanol can also be oxidized in the cytosol of acetic acid bacteria by two nicotinamide adenine dinucleotide (NAD)-dependent enzymes, alcohol dehydrogenase (NAD-ADH) and aldehyde dehydrogenase (NAD-ALDH). Subsequently, acetate is converted into acetyl-CoA and, via tricarboxylic acid (TCA) cycle, acetate is metabolized into carbon dioxide and water, as shown in Figure 3 [Mamlouk *et al.*, 2013]. Additionally, *Acetobacter*, *Gluconacetobacter*, *Komagataeibacter* and *Acidomonas* species are capable of oxidizing ethanol completely to carbon dioxide and water, whereas oxidation of acetate is weak in *Asaia* and

Kozakia species and absent in *Gluconobacter* strains, due to a lack of activity of two enzymes of the TCA cycle, α-ketoglutarate-dehydrogenase and succinate dehydrogenase.

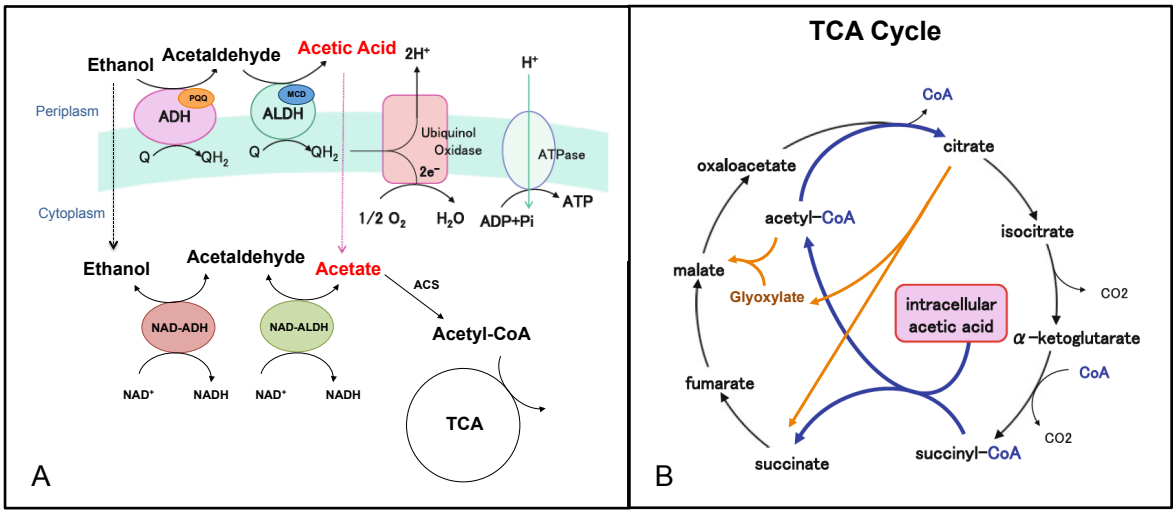


Figure 3. Representation of ethanol oxidation of acetic acid bacteria, both at a periplasmic level (incomplete oxidation) and cytoplasmic level (complete oxidation). A: Ethanol and acetate oxidations in acetic acid bacteria, coupled with ATP generation. B: Tricarboxylic acid (TCA) of acetic acid bacteria, showing how acetic acid is assimilated. Both figures were adapted from Matsushita *et al*. (2016).

The sequential oxidations of ethanol and acetate leads to a diauxic growth nature, characterized by the accumulation of acetic acid in the first exponential growth phase, a transition phase and lastly, the second exponential growth phase, where acetic acid is oxidized into carbon dioxide and water, as demonstrated in Figure 4.

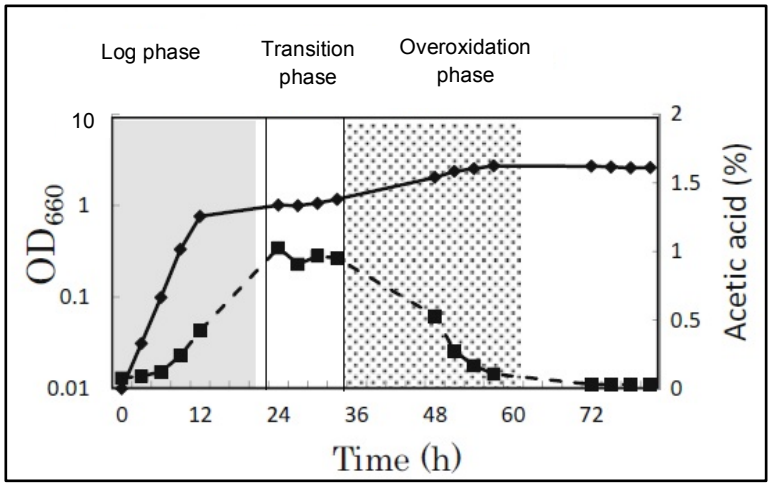


Figure 4. Representation of a typical diauxic growth curve of acetic acid bacteria capable of oxidizing acetic acid to carbon dioxide and water (complete oxidation). The full line (◆) represents the optical density (OD) measured at 660 nm and the dotted line (■) represents the concentration of acetic acid in the culture medium. Extracted from Matsushita *et al*. (2016).

Most of these metabolic features of acetic acid bacteria are still poorly characterized. Recent studies of whole genome sequencing and transcriptome analysis have shed some light on this matter, at least in *Acetobacter aceti* [Matsushita *et al*., 2016; Sakurai *et al*., 2012]. Carbon flow through the TCA cycle of acetic acid bacteria is generally reduced during the incomplete oxidation of ethanol. The low flux rate of the TCA cycle in the presence of ethanol causes cytoplasmic accumulation of acetate and/or acetyl-CoA. It seems that *A. aceti* has at least two different mechanisms to convert acetate into acetyl-CoA, namely i) conversion of acetate into acetyl-CoA by acetyl-CoA synthase (ACS); ii) conversion of succinyl-CoA to succinate and simultaneously transfer of CoA to acetic acid. In the absence of ethanol, acetyl-CoA can be incorporated into the TCA cycle by either its conversion into citrate or into malate (though the glyoxylate pathway, although not all strains exhibit an active glyoxylate pathway). Thus, it seems that these bacteria may have a particularly efficient system to consume intracellular acetate. The consumption of acetate through the TCA cycle generates reducing power in the form of NADH, which is reoxidized to NAD^+ by membrane NADH dehydrogenases (with or without proton translocating ability) [Sakurai *et al*., 2012].

Ethanol is nearly stoichiometrically converted to acetic acid. During the oxidation of ethanol into acetic acid, the TCA cycle functions only to meet the demands for synthesis of biomass and ethanol is used exclusively as an energy source [Sakurai *et al*., 2012]. The glyoxylate pathway functions as an anaplerotic shunt and allows the conversion of acetate into biomass through the synthesis of precursors, such as oxaloacetate, pyruvate and phosphoenolpyruvate [Adler *et al*., 2014]. However, the glyoxylate pathway is not a requirement for the growth of acetic acid bacteria. Strains which do not exhibit an active glyoxylate shunt can synthetize oxaloacetate from amino acids and other compounds contained in organic substrates, present in wine mostly due to the autolysis of yeast cells [Matsushita *et al*., 2016]. Glycerol is a major byproduct of alcoholic fermentation. Although most of the glycerol is oxidized to dihydroxyacetone, some may be used as a carbon source for the production of biomass [Guillámon *et al*., 2011]. Interestingly, acetic acid bacteria isolated from cocoa beans fermentation have been shown to strictly utilize ethanol for the generation of metabolic energy through acetate production, while lactate is mainly used for the generation of biomass through gluconeogenesis and pentose phosphate pathways [Adler *et al*., 2014]. When grown in (red) wine, acetic acid bacteria may use glycerol, lactate and other organic compounds as carbon sources for the generation of biomass.

Acetic acid resistance. Acetic acid resistance is a crucial factor in the production of large amounts of acetic acid, a weak acid well known for its ability to inhibit growth of most microorganisms at very low concentrations (0.5%). Acetic acid is a lipophilic molecule that can easily diffuse through the cytoplasmic membrane, where the toxic effect is caused by its dissociation in the higher pH of the cytoplasm. The released protons lower the pH of the cytoplasm, resulting in the denaturation of various proteins and the disruption of the membrane electro-chemical gradient [Matsushita *et al*., 2016].

The mechanisms of acetic acid bacteria that confer resistance or adaptation to acetic acid are not completely understood yet. Although some resistance mechanisms have been reported in recent years, much of the knowledge necessary for an extensive understanding of the acetic acid resistance demonstrated by this group of bacteria is still nonexistent. Acetic acid resistance is species and strain dependent, with strains rapidly losing their resistance to high concentrations of acetic acid on synthetic media under laboratory conditions. This seems to indicate that the molecular mechanisms involved in acetic acid resistance are inducible and transient [Raspor *et al*., 2008]. Nevertheless, at least four methods of resisting acetic acid have been reported for acetic acid bacteria, namely i) prevention of acetic acid influx into the cell by alteration of components of the lipid membrane and the establishment of polysaccharides on the cell surface; ii) efficient acetic acid assimilation through the TCA cycle; iii) acetic acid efflux by expression of a putative ATP-binding cassette transporter; and iv) protection of cytosolic proteins against denaturation by expression of chaperones [Matsushita *et al*., 2016; Raspor *et al*., 2008]. The complete understanding of the metabolic pathways that confer resistance to acetic acid can lead to the selection of bacteria with unique physiological properties, enabling an improvement of wine vinegar production, as well as other natural "fermentation" processes.

Nitrogen fixation. Nitrogen fixation, or diazotrophy, is the capacity to fix free molecular nitrogen (N_2) into cell material by its reduction to ammonium. Among acetic acid bacteria, different genera and species are capable of fixating molecular nitrogen, namely, *Gluconacetobacter diazotrophicus*, *Ga. johannae*, *Ga. azotocaptans*, *Swaminathania salitolerans*, *Acetobacter peroxydans*, and *A. nitrogenifigens* [Matsushita *et al*., 2016; Sievers & Swings, 2005]. Since optimal nitrogen fixation by *Ga*. *diazotrophicus* demands high aerobic conditions for efficient energy production, a capable protection mechanism is necessary to protect nitrogenase activity from the damaging action of oxygen. It is suggested that this organism uses a respiratory protection mechanism to maintain its nitrogenase activity in the presence of oxygen [Raspor *et al*., 2008].

1.1.4 Biotechnological Applications

People have taken advantage of the unique metabolism of acetic acid bacteria long before they were acknowledged as the causative agent of the "acetic acid fermentation". Recently, their physiology has been extensively studied due to the innumerous possibilities of exploitation of their oxidation machinery [Mamlouk *et al*., 2013]. Currently, their biotechnological applications have been widely increased beyond their role in the vinegar industry. Despite the occurrence of acetic acid bacteria being detrimental in some cases, they mostly perform a positive role in many bioprocesses.

1.1.4.1 Wine Vinegar

The word "vinegar" is originated from the French words "vin" and "aigre", which literally mean "sour wine". Wine and vinegar production have always been linked and there are reports of its utilization as early as 4000 B.C. Vinegar can be defined as a solution of acetic acid and a product of "acetic acid

fermentation" from alcoholic solutions (ranging from 10% to 15% of ethanol). The most common raw materials for its production are wine, beer, cider and products from alcoholic fermentations of numerous cereals and fruits [Komagata *et al*., 2014]. Despite its starting raw material, vinegar is produced by a two-step process: firstly, ethanol is produced by yeasts from a carbohydrate, such as sugars or starch (firstly, starch should be broken down into glucose or maltose), and then the oxidation of ethanol into acetic acid is performed by acetic acid bacteria, mainly from the genera *Acetobacter*, *Gluconobacter* and *Komagataeibacter* [Matsushita *et al*., 2016].

Traditionally, vinegar is produced by a process called surface fermentation, which consists of a static culture of acetic acid bacteria at the air-liquid interface. It is usually performed in wooden barrels (different types of wood can be used) filled to 2/3 of their capacity to create an air chamber, connected to the outside air by various types of openings. The acetic acid bacteria form a biofilm on the liquid surface, generally called "mother of vinegar", composed of cellulose and possibly of other exopolysaccharides. Because it floats, this cellulosic matrix allows acetic acid bacteria to position at the air-liquid interface, giving them access to the atmospheric oxygen in the air phase and the nutrients required for growth in the liquid phase. It is thought that this biofilm also confers protection against harmful chemicals (may also protect against high acetic acid and ethanol concentrations) and dehydration and supports cell-to-cell contact [Matsushita *et al*., 2016].

Vinegars produced by this process are considered to be of high quality due to their organoleptic complexity. Along with the oxidation of ethanol to acetic acid, the secondary metabolism of acetic acid bacteria results in addition of flavor and aroma to the final product, mostly through oxidation reactions, but also through esterification reactions. Because of this, the microorganisms producing the vinegar have a high impact on the quality of the vinegar. Additionally, the intrinsic properties of the raw material and the interaction with the wood from the barrels generates vinegars with a variety of odor, taste, color and other properties [Mas *et al*., 2014]. This production method can take as long as 2 years [Matsushita *et al*., 2016].

In the early 50's, submerged culture processes were introduced, mainly for the production of antibiotics. These processes were carried out in stainless steel bioreactors that were promptly applied to the vinegar industry. The bioreactors are equipped with devices that ensure a continuous and homogeneous airflow, thermometers and cooling systems for monitoring and maintaining liquid temperature at 30ºC, an automatic device to measure the alcohol content and a mechanical defoamer [Gullo *et al*., 2014].

One of the most important aspects of submerged fermentation is the oxygenation of the liquid. It is described that an interruption of aeration of only a few minutes can lead to a complete arrest of acetification that cannot be recovered on its own when aeration is restarted. Under industrial conditions, acetic acid bacteria undergo considerable stress from both high ethanol content (acetification usually starts with concentrations of 7% to 15%) and high acetic acid content (at the end of acetification, concentrations of 10% to 12% are reached). In order to survive under these stressful conditions, the bacteria need a constant supply of energy from respiration [Gullo *et al*., 2014; Raspor *et al*., 2008].

Submerged fermentation at an industrial scale is commonly operated in semi-continuous mode. This production process is advantageous because it reduces the risk of substrate inhibition and allows the reuse of the microbial culture in the subsequent production cycle [Gullo *et al*., 2014]. Each cycle takes 24 to 48 hours. When alcohol concentration reaches a minimum residual level, a portion of the vinegar is removed, normally around 2/3 of the bioreactor, and is replaced with fresh mash (wine). It is particularly important to monitor ethanol levels because in case of ethanol depletion, *Acetobacter* and *Komagataeibacter* strains begin to oxidize acetic acid to carbon dioxide and water. Since ethanol represses this change in metabolism, a residual level of ethanol is always maintained [Raspor *et al*., 2008]. The highest reported production yields in submerged fermentation were obtained with this operation mode [Gullo *et al*., 2014]. The downstream processing of vinegar production is composed of several steps, such as cell separation, sedimentation, clarification, pasteurization, filtration, and dilution [Gullo *et al*., 2014].

The submerged fermentation, in comparison with the surface fermentation, results in higher productivity, faster conversion of ethanol to acetic acid and lower capital investment per product amount [Raspor *et al*., 2008]. Remarkably, it is still state of the art, in both operating methods, to start the acetification process with a microbiological undefined culture [Sokollek *et al*., 1998].

Acetic acid bacteria as starters. In industrial vinegar production, there are several circumstances that can lead to a complete arrest of acetification, such as abrupt temperature changes when loading or unloading the acetator, interruption of aeration and infection with bacteriophages. This breakdown of the acetification process can take up to several days or weeks to restart. Therefore, the availability of acetic acid bacteria starter cultures is extremely desirable for the vinegar industry to improve the process control [Sokollek *et al*., 1997].

The utilization of an undefined microbial community as a starter culture by this industry is a reflection of the problems with acetic acid bacteria isolation, culture maintenance, cultivation outside the acetator, transfer from liquid to solid media, determination of viable counts, strain preservation and loss of phenotype over multiple cultivation cycles [Gullo *et al*., 2014; Sokollek *et al*., 1997].

Although some studies have tried to evaluate and develop optimized acetic acid bacteria as starter cultures, these efforts have not met the industrial demand for stable and robust strains. The characteristics described for an optimal starter strain include high acetic acid production yield, high tolerance to ethanol and acetic acid, low nutrient requirements, low pH resistance, thermotolerance, no cellulose production, resistance to bacteriophages and addition of enhanced organoleptic attributes [Gullo *et al*., 2014; Matsushita *et al*., 2016].

Therefore, typification, identification and determination of phenotypic traits of indigenous acetic acid bacteria isolates is a basis for the search of functional starters that serve as a means of increasing the productive capacity of this industry, by accelerating the start of the acetification process, enabling culture rotation as a way for controlling phage complications and enhancing the quality and standardization of the final product.

1.1.4.2 Other Types of Vinegar

Additionally to wine vinegar, acetic acid bacteria are used to produce a notable variety of vinegars, depending on the raw material used. The common types of vinegar within a region habitually reflect the local alcoholic beverage [Raspor *et al*., 2008].

Cider vinegar. Traditionally, cider vinegar was produced with apple juice or concentrated apple juice through a co-fermentation by indigenous yeasts and acetic acid bacteria, with the co-production of ethanol and acetic acid. Nowadays, cider vinegar is mostly produced by submerged fermentation and is mainly used in western countries as a table vinegar and as a preservative. Studies show that *Km. europaeus* is the dominant species in submerged fermentation. However, cider vinegars usually show a higher species diversity within acetic acid bacteria than wine or spirit vinegars, probably due to its higher content in sugars (4%) and lower content in ethanol (6%), while wine vinegar (12% ethanol) and spirit vinegar (14% ethanol) exert more stressing conditions for bacterial growth [Férnandez-Pérez *et al*., 2010; Matsushita *et al*., 2016; Raspor *et al*., 2008].

Balsamic vinegar. Traditional balsamic vinegar (TBV), a highly prized vinegar, has been produced in Northern Italy for centuries, in the regions of Modena and Reggio Emilia. The grape must is gently boiled for several hours, until it has reduced to about one-half or one-third of its initial volume, resulting in a liquor with a high sugar concentration (around 30%), where both alcoholic fermentation and acetification take place. This liquor is transferred to a series of barrels, with decreasing volumes arranged in succession, composed of different woods (ash, cherry, oak, juniper, mulberry and chestnut), with each wood influencing the vinegar in a unique way. Traditionally, part of the contents of the smallest barrel are collected each year (the finalized TBV) and are replaced with the same volume from the preceding barrel, and so on up the line, until the biggest barrel is filled with the cooked must of that season. This method takes at least twelve years and its not uncommon to find TBV with fifty or more years. Although this process has a very low yield, the resulting vinegar is of exceptionally high quality, with a dark brown color, a dense consistency, both sweet and sour and very complex in taste [Matsushita *et al*., 2016; Raspor *et al*., 2008].

Acetic acid bacteria inhabiting TBV are not well known, with only a few ecological studies reported. Nevertheless, some strains have been isolated from TBV and identified, particularly strains belonging to the following species: *Km. europaeus*, *Km. xylinus*, *Km. hansenii*, *A. pasteurianus*, *A. aceti* and *A. malorum* [Gullo *et al*., 2008].

Spirit vinegar. This type of vinegar, sometimes referred to as white, distilled or alcohol vinegar, is characterized by a high acetic acid content (up to 15%) and is not very aromatic. Its raw material is diluted purified ethanol or, in countries where it is permitted by law, it can be produced from synthetic ethanol. It has a low cost production and is widely spread throughout the world. It is commonly sold completely colorless or with a yellowish color obtained by the addition of caramel or other food colorants. Strains of two acetic acid bacteria species have been isolated from this type of vinegar, *Km.*

intermedius and *Km. oboediens*. However, no growth was observed in any culture media when samples of high-acidic vinegar (> 10%) were plated, suggesting that the main acetic acid producer is yet to be disclosed [Matsushita *et al*., 2016; Raspor *et al*., 2008].

Rice vinegar. In the Far East, mostly in China, Taiwan and Japan, vinegar has been produced from polished rice (*komesu*) and unpolished rice (*kurosu*) for several centuries. *Komesu* is colorless and has a plain taste, traditionally used for sushi cooking, while *kurosu* is black, containing more amino acids and vitamins than *komesu* and is usually used as a healthy drink. The final acidity of these vinegars is typically less than 10%. Both these vinegars were traditionally produced by surface fermentation but this technique has been largely replaced by submerged fermentation methods. The acetic acid bacteria community of a traditional surface fermentation process was investigated. Almost all strains isolated from four different phases of production corresponded to *A. pasteurianus* and the authors concluded that over 100 years of vinegar production, an almost pure culture of acetic acid bacteria was maintained [Matsushita *et al*., 2016; Nanda *et al*., 2001].

1.1.4.3 Bacterial Cellulose

Some acetic acid bacteria have the ability to produce cellulose, a polymer of β-1,4-linked glucose units. *Km. xylinus* is world widely regarded as the model organism for studying bacterial cellulose biosynthesis. Although the chemical structure of bacterial and plant cellulose is identical, the physical structure of bacterial cellulose is quite unique, since it is formed by ultrafine fibers that form an ultrafine network. This results in excellent properties, such as enormous mechanical strength, hydrophilicity (can hold a large amount of water, 200 times its dry mass), great elasticity and conformability (can be molded into any shape and size during its synthesis), high purity and it's biocompatible and biodegradable [Matsushita *et al*., 2016; Raspor *et al*., 2008].

From an industrial point of view, bacterial cellulose has a huge biotechnological potential, with many patent applications submitted all over the world, but only a few commercial applications currently available. One of the first applications of bacterial cellulose is its use as an acoustic transducer diaphragm and has been commercialized in various types of speaker units and headsets. Another commercialized application of this biopolymer is in the medical biotechnology field, where cellulose is used as temporary wound dressing or artificial skin for patients with burns, chronic skin ulcers or other extensive loss of tissue. The biocompatibility and high water retention demonstrated by bacterial cellulose seems to stimulate growth of the skin tissue, resulting in a faster healing rate with a lower risk of infection and reduced treatment time and cost. Two other potential applications for bacterial cellulose in this field are being explored, namely, using cellulose as artificial blood vessels, since it carries a low risk for blood clot development and has great shape retention and tear resistance; and as a scaffold for tissue engineering, since bacterial cellulose supports the growth of mammalian cells and can assume any shape for the new growing tissue.

However, current methodologies for bacterial cellulose synthesis are still far from a large-scale production, resulting in a high production cost with a low yield [Matsushita *et al*., 2016; Raspor *et al*., 2008].

In addition to vinegar and cellulose production, acetic acid bacteria have been extended to a number of industries like food production (*nata de coco* and cocoa), pharmaceutics (L-ascorbic acid), biotransformations and fine chemicals production (D-tagatose and shikimate), and their fields of application are only expected to increase [Raspor *et al*., 2008].

1.2 Thesis Scope and Goals

This thesis was planned in association with the project BIOPEPPERtec (ANI Proposal 3321), in a partnership with Mendes Gonçalves S.A., a major Portuguese vinegar producing company, among other products, located in Golegã. Although the project was approved, no financing was attributed for the Lisbon located partner (FCUL) and so it was withdrawn. Nevertheless, the research partnership between Mendes Gonçalves and Lab Bugworkers | M&B-BioISI was maintained and thus this thesis could be carried out.

One of the limitations of the vinegar industry is the loss of viability and/or productivity of acetic acid bacteria when an alteration of the raw material is made. Also, the undefined microbial community used as inoculum necessarily undergoes variations, in terms of species diversity and proportion, due to the cyclic nature of the process. The inexistence of fine monitoring methodologies and the unavailability of starter cultures at Mendes Gonçalves result in a limitation in the application of prophylactic or corrective measures in the acetification process. In light of these difficulties associated with the industrial production of vinegar, several objectives were proposed for this thesis:

i) creation of a collection of acetic acid bacteria isolated from vinegars produced at Mendes Gonçalves;
ii) molecular characterization and identification to the species level of the isolated strains;
iii) physiological characterization of the identified strains;
iv) development of molecular methodologies to be applied in the monitoring of industrial biotechnological processes at Mendes Gonçalves.

2. Methods

2.1 Vinegar Samples and Bacterial Strains

Forty-one samples of vinegars produced/processed by Mendes Gonçalves (MG) were brought to the Lab Bugworkers | M&B-BioISI. These samples consisted of different vinegars, such as red and white wine, balsamic, cider, cereal, spirit, rice, vinegars aromatized with several fruits and vinegars aged in oak barrels, with acidity levels ranging from 5% to 8%. These vinegar samples were screened for acetic acid bacteria using two culture-based methods. Five additional red wine vinegar samples were obtained from MG. These five samples were collected from the same acetator, throughout 36 hours, corresponding to different stages of a red wine vinegar production cycle.

A total of 31 acetic acid bacteria strains were used in this study, belonging to different genera and corresponding to 22 wild strains and 9 strains obtained from culture collections. The wild strains were isolated both in the MG microbiology laboratory (16 isolates) and in the Lab Bugworkers | M&B-BioISI (6 isolates), from different types of vinegar.

The type strains of 7 acetic acid bacteria species were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). These strains were reactivated according to the DSMZ recommendations. Two additional strains were acquired from the Spanish Type Culture Collection (CECT) and were reactivated according to the CECT recommendations. These 9 strains acquired from culture collections were used in this study as reference strains (Table 2). All strains (wild and reference) were cultivated on GYC plates (with the exception of *Km. europaeus* DSMZ 6160^T that was cultivated on Sabouraud plates, supplemented with 0.32% acetic acid, according to DSMZ recommendations). Acetic acid bacteria isolates and reference strains were stored at -80ºC, in a solution of RAE medium and glycerol as a cryoprotectant (at a final concentration of 20%).

Table 2. List of strains used in this study as reference strains. DSMZ: German Collection of Microorganisms and Cell Cultures; CECT: Spanish Type Culture Collection. *Strain CECT 824 was wrongly identified as *Acetobacter pasteurianus* and its identity was determined with the amplification and sequencing of a portion of the 16S rRNA gene (as described in section 2.4).

Species	Isolation Source	Strain Name
Acetobacter aceti	Alcohol turned to vinegar	DSMZ 3508^T
*Acetobacter cerevisiae**	Turbid beer	CECT 824
Acetobacter pasteurianus	Fermented beverages (beer)	DSMZ 3509^T
Gluconobacter oxydans	Not available	CECT 4009
Gluconobacter oxydans	Fermented beverages	DSMZ 3503^T
Komagataeibacter europaeus	Submerged vinegar production	DSMZ 6160^T
Komagataeibacter hansenii	Vinegar	DSMZ 5602^T
Komagataeibacter intermedius	Tea fungus beverage (*kombucha*)	DSMZ 11804^T
Komagataeibacter xylinus	Mountain ash berries	DSMZ 6513^T

2.2 Isolation of Acetic Acid Bacteria

The vinegar samples were analyzed for acetic acid bacteria using two culture methods, a direct approach and/or an enrichment approach. The compositions of the culture media used in this study are presented in annex A1.

The direct approach consisted in directly plating 100 µl of each vinegar sample on GYC plates. For the enrichment approach, 15 ml of each sample was centrifuged at 3220xg for 15 minutes in a Centrifuge 5810 R (Eppendorf, Germany) and the cellular pellet was inoculated in 20 ml of GYP medium, supplemented with 3% ethanol (v/v). The liquid cultures were incubated at 28ºC and 160 rpm for 5 days. Then, the total volume of each culture was centrifuged in the same conditions described above and the cellular pellet was plated on GYC plates.

All plates were incubated at 28ºC and were checked for colony growth at day 3, 5 and 7. Every acid-producing colony and every colony showing different morphological characteristics were purified by streak-plating and subjected to further characterization (Gram, KOH, catalase and oxidase tests).

2.3 Genomic Fingerprinting: RAPD-PCR, REP-PCR and ERIC-PCR

Genomic DNA was extracted from bacterial cells in a pure culture using an adapted Guanidium Thiocyanate method described by Pitcher et al. (1989). These modifications were made primarily in the first steps of the method. Bacterial cells collected from an agar plate were ressuspended in 250 µl of lysis buffer (50 mM Tris; 250 mM NaCl; 50 mM EDTA; 0.3% SDS; pH 8.0) and 100 µl of microspheres. After 2 minutes of homogenization in a vortex, the cells were incubated in 65ºC for 30 minutes, followed by another 2 minutes of homogenization. Afterwards, the GES reagent (5 M guanidium thiocyanate; 10 mM EDTA; 0.5% Sarkosyl; pH 8,0) was added and the original method was followed but using an equal volume of isopropanol.

The extracted DNA was visualized by electrophoresis in 0.8% (w/v) agarose gels using a 1kb Plus DNA ladder (Invitrogen). The electrophoresis was performed in 0.5x TBE buffer with a constant voltage of 4.5 V/cm. Afterwards, the gel was stained in an ethidium bromide solution and photographed in an Alliance 4.7 UV transiluminador (UVItec, Cambridge) using the Alliance software (version 15.15, UVItec, Cambridge).

Genomic DNA, extracted from bacterial isolates in pure cultures, was used as template to obtain genomic fingerprints. Three different PCR fingerprinting methods were applied: i) Randomly Amplified Polymorphic DNA (RAPD-PCR); ii) Repetitive Extragenic Palindromic sequences (REP-PCR) and iii) Enterobacterial Repetitive Intergenic Consensus sequences (ERIC-PCR). The RAPD-PCR was performed using the PH primer (5' AAGGAGGTGATCCAGCCGCA '3) [Massol-Deva *et al*., 1995] and the REP-PCR was performed using the GTG_5 primer (5' GTGGTGGTGGTGGTG '3) [De Vuyst *et al*., 2008]. Both amplification reactions were carried out in a total volume of 25 µl, containing 1x PCR reaction buffer, 3 mM of $MgCl_2$, 25 pmol of primer, 0.2 mM of each of the four dNTP's, 1 U of

Taq polymerase and 1 µl of template DNA per reaction. All reagents used were acquired from Invitrogen (Massachusetts, USA). This assay was performed in a UNO II thermal cycler (Biometra, Germany), with the following PCR conditions: 5 min of initial denaturation at 95ºC, followed by 40 cycles of denaturation at 95ºC for 1 min, annealing at 50ºC for 2 min and extension at 72ºC for 2 min, and a final extension at 72ºC for 5 min.

The ERIC-PCR was performed using the pair of primers ERIC-1 (5' ATGTAAGCTCCTGGGGATTCAC '3) and ERIC-2 (5' AAGTAAGTGACTGGGGTGAGCG '3) [Versalvoice *et al*., 1991]. The amplification reaction was carried out in a total volume of 25 µl, containing 1x PCR reaction buffer, 3 mM of $MgCl_2$, 25 pmol of each of the ERIC primers, 0.2 mM of each of the four dNTP's, 1 U of *Taq* polymerase and 1 µl of template DNA per reaction. All reagents used were acquired from Invitrogen (Massachusetts, USA). This assay was performed in a UNO II thermal cycler (Biometra, Germany), with the following PCR conditions: 5 min of initial denaturation at 95ºC, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 57ºC for 1 min and extension at 72ºC for 2 min, and a final extension at 72ºC for 5 min. The products of the amplification reactions described above were visualized by electrophoresis in a 1.2% (w/v) agarose gel using a 1kb Plus DNA ladder (Invitrogen). The electrophoresis was performed in 0.5x TBE buffer with a constant voltage of 2.5 V/cm. The gel was stained in an ethidium bromide solution and photographed in an Alliance 4.7 UV transiluminador (UVItec, Cambridge) with Alliance software (version 15.15, UVItec, Cambridge).

The banding patterns were analyzed with Bionumerics software (version 6.6, Applied Maths) and a composite dendrogram was created based on the genomic profiles obtained with the primers GTG_5 and PH. This dendrogram was constructed using the Pearson correlation coefficient as a similarity measure and the unweighted pair group method with the arithmetic average clustering algorithm (UPGMA). A reproducibility assay was performed to determine the percentage of similarity necessary for strain discrimination. For each type of genomic fingerprinting, 10% of the isolates (3) were randomly chosen and the amplification reaction was performed in duplicate. A dendrogram was built for these three isolates and their repeats and the optimization parameters were adjusted until each isolate was grouped with its repeat. The reproducibility of each type of genomic fingerprinting was determined as the average of the levels of similarity observed between repeats. The reproducibility of the composite dendrogram was defined as the average of the reproducibilities determined for each type of genomic fingerprinting.

2.4 Molecular Identification by 16S rRNA Gene Sequencing

DNA from bacterial isolates (DNA extraction described in section 2.3) was used as a template for the amplification of a portion of the 16S rRNA gene, using the universal primers PA (27f) (5' AGAGTTTGATCCTGGCTCAG 3') [Massol-Deva *et al*., 1995] and 907r (5' CCGTCAATTCMTTTRAGTTT 3') [Muyzer *et al*., 1998].

The amplification reaction was carried out in a total volume of 50 µl, containing 1x PCR reaction buffer, 2 mM of $MgCl_2$, 25 pmol of each primer, 0.2 mM of each of the four dNTP's, 1 U of *Taq*

polymerase and 1 µl of template DNA per reaction. All reagents used were acquired from Invitrogen (Massachusetts, USA). This assay was performed in a UNO II thermal cycler (Biometra, Germany), with the following PCR conditions: 5 min of initial denaturation at 95ºC, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min and extension at 72ºC for 2 min, and a final extension at 72ºC for 5 min.

The products of the amplification reaction described above were visualized by electrophoresis in a 1.2% (w/v) agarose gel using a 1kb Plus DNA ladder (Invitrogen). The electrophoresis was performed in 0.5x TBE buffer with a constant voltage of 4.5 V/cm. The gel was stained in an ethidium bromide solution and photographed in an Alliance 4.7 UV transiluminador (UVItec, Cambridge) with Alliance software (version 15.15). Only 5 µl of the reaction were loaded in the agarose gel, while the remaining volume was purified using the kit Jet Quick PCR Product Purification Spin Kit (Genomed, USA), following the manufacturer's recommendations. The purified PCR products were sequenced by Biopremier (Lisbon, Portugal) and the algorithm BLAST (Basic Local Alignment Search Tool) was used to determine the closest known relative(s) of the partial 16S rRNA sequence obtained. Additionally, a phylogenetic reconstruction was generated using the MEGA software (version 7.0.20). The 16S rRNA gene sequences obtained were aligned (ClustalW 1.6) with the sequences of the same gene of the type strains of all species of the genus *Komagataeibacter* and were clustered with the neighbor-joining algorithm.

2.5 Multiplex-PCR: Primer Design and PCR Conditions

Four different degenerate primers were designed based on DNA sequences available in GenBank for the subunit I of the PQQ-dependent ADH gene, *adhA*. The multiple alignment was done using the algorithm ClustalW (version 1.6) and the primers were designed based on the conserved regions shown by the alignment. Degenerate bases were chosen in positions where all of the DNA sequences did not align and bases with the lowest degree of the degeneracy were preferred. All primers are composed of 20 nucleotides, consisting in two forward primers (ADH-F1 and ADH-F2) and two reverse primers (ADH-R1 and ADH-R2) (Table 3). Therefore, there are four possible combinations of primers, resulting in four different fragments with sizes of 240 bp (primers ADH-F2 and ADH-R2), 336 bp (primers ADH-F1 and ADH-R2), 388 bp (primers ADH-F2 and ADH-R1) and 484 bp (primers ADH-F1 and ADH-R1).

Table 3. List of the *adhA* directed primers.

Name	Position	Sequence
ADH-F1	5-24	5' ACMGCNACATACTGCTTGCC 3'
ADH-R1	470-489	5' TGGTACGGCATKCCSGGKGA 3'
ADH-F2	101-120	5' GCGTCRTARGCRTGGAATTC 3'
ADH-R2	322-341	5' TKGGYCTSGACATGAACAAG 3'

Different conditions were tested in order to optimize the PCR amplification. A PCR assay was planned where two concentrations of $MgCl_2$ (1.5 mM and 2.5 mM) and primers (25 pmol and 50 pmol) and a range of annealing temperatures (46.5ºC to 57.5ºC) were tested for the four possible primer combinations.

The final amplification reaction was performed in a total volume of 25 µl, containing 1x PCR reaction buffer, 1.5 mM of $MgCl_2$, 25 pmol of each of the *adhA* directed primers (ADH-F1, ADH-F2 and ADH-R2), 6.25 pmol of each of the 16S rRNA gene directed primers (PA and 907r), 0.2 mM of each of the four dNTP's, 1 U of *Taq* polymerase and 1 µl of template DNA per reaction. All reagents used were acquired from Invitrogen (Massachusetts, USA). This assay was performed in a T Gradient thermal cycler (Biometra, Germany), with the following PCR conditions: 5 min of initial denaturation at 95ºC, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 57ºC for 1 min and extension at 72ºC for 1 min, and a final extension at 72ºC for 5 min.

The amplification products were visualized by electrophoresis in a 1.2% (w/v) agarose gel using a 1kb Plus DNA ladder (Invitrogen, Massachusetts, USA). The electrophoresis was performed in 0.5x TBE buffer with a constant voltage of 4.5 V/cm. The gel was stained in an ethidium bromide solution and photographed in an Alliance 4.7 UV transiluminador (UVItec, Cambridge) using the Alliance software (version 15.15).

2.6 Growth Analysis and Quantification of Acetic Acid

Acetic acid bacteria strains were grown in red wine (14.4% ethanol) supplied by Mendes Gonçalves, where the ethanol content of the wine was determined. The wine was centrifuged at 15000xg for 10 minutes in a J2-21 centrifuge (Beckman, USA) and the supernatant was sterilized by filtration with a 0.45 µm filter. The wine was diluted in autoclaved ultrapure water in a proportion of 1:2, respectively (the final ethanol concentration was expected to be around 4.8%). Pre-cultures were grown (28ºC, 160 rpm) during four days, in 100 ml Erlenmeyers with 50 ml of GYP medium, supplemented with 2% ethanol and inoculated with a loopfull (10 µl) from frozen (-80ºC) preparations of the strains. After four days, each pre-culture was centrifuged at 3220xg for 15 minutes in a Centrifuge 5810 R (Eppendorf, Germany) and the cellular pellet was ressuspended in 300 µl of GYP medium. For each strain, three 250 ml Erlenmeyers with 100 ml of the diluted wine were inoculated with 100 µl of the pre-culture ressuspended pellet. The cultures were incubated for several days at 28ºC and 160 rpm and samples were collected at several time-points. At each time-point, 1 ml of the culture was collected to a cuvette to measure the optical density (OD). Additionally, another culture sample of 1 ml was collected and centrifuged at 17968xg for 10 minutes in a 1-15P centrifuge (Sigma, USA). Subsequently, 900 µl were collected to another tube and frozen at -20ºC until they were used to measure the pH and the acetic acid concentration.

Analytical Methods. OD was measured using a UV1101 Biotech Photometer (WPA, UK) with a 600 nm filter and the pH was measured using a BioTrode lab pH microelectrode (Hamilton, Switzerland) coupled with a model 15 pH meter (Denver Instruments, USA). Acetic acid concentration was determined enzymatically, using the Acetic Acid Assay Kit (Acetate Kinase Manual Format) from Megazyme (Ireland). All reactions were performed in microplate assay and serial dilutions of the samples were prepared when necessary. A calibration curve was constructed within the linearity reported by the manufacturer (0.1-2.5 µg of acetic acid per well) and is presented in annex A3.

Data analysis. The specific growth rate (µ) was calculated based on a regression the logarithm of the OD values during exponential growth phase. The acetic acid production rate was calculated based on a regression of the acetic acid concentrations from the start of acetic acid production until the maximum concentration was obtained. Linearity of all regressions was assessed by the determination coefficient values (R^2).

Additionally, the maximal bioconversion efficiency of acetic acid was calculated as described by [Mounir *et al.*, 2016]. For this calculation, it was assumed that the initial concentration of ethanol was 4.8% (v/v) [equivalent to 3.7% (w/v)] and the final concentration of ethanol was 0.0% (v/v). The productivity ($gl^{-1}day^{-1}$) of acetic acid production was calculated according to the following equation:

$$P = \frac{[AcH]_{max} - [AcH]_i}{t}$$

where $[AcH]_{max}$ is the maximum concentration (gl^{-1}) of acetic acid obtained, $[AcH]_i$ is the initial concentration (gl^{-1}) of acetic acid and t corresponds to the time (days) at which the maximum concentration of acetic acid was obtained.

2.7 Microbial Profiling Analysis

Community DNA was extracted from the five red wine vinegar samples using the DNA Isolation Kit PowerMax™Soil (MO BIO Laboratories, USA). About 250 ml of each vinegar sample was centrifuged at 15000xg for 10 minutes in a J2-21 centrifuge (Beckman, USA) and the supernatant was discarded. The cellular pellet was transferred directly to the DNA extraction kit and manufacturer's recommendations were followed.

The DNA extracts were provided to Biopremier (Lisbon, Portugal) where the Microbial Profiling analysis was performed. Using the community DNA extracts as a template, two different regions of the 16S rRNA gene were amplified by PCR (region 1 and region 2). Region 1 corresponds to a fragment of around 300 nucleotides, situated in the 5' end of the gene and region 2 corresponds a fragment of around 200 nucleotides, situated in the 3' end of the gene. Primers with different tags were used for the different regions and for the different time-points. Afterwards, the amplified fragments were pooled together and sequenced by NGS technology (Ion Torrent) and the resulting DNA sequences were identified by a BLAST analysis.

3. Results and Discussion

3.1 Isolation of Acetic Acid Bacteria

In this study, forty-one samples of vinegars produced and/or processed at Mendes Gonçalves were analyzed using two culture-based methods, a direct approach and/or an enrichment approach, with the objective of isolating acetic acid bacteria. Figure 5 shows the results obtained with both culture methods.

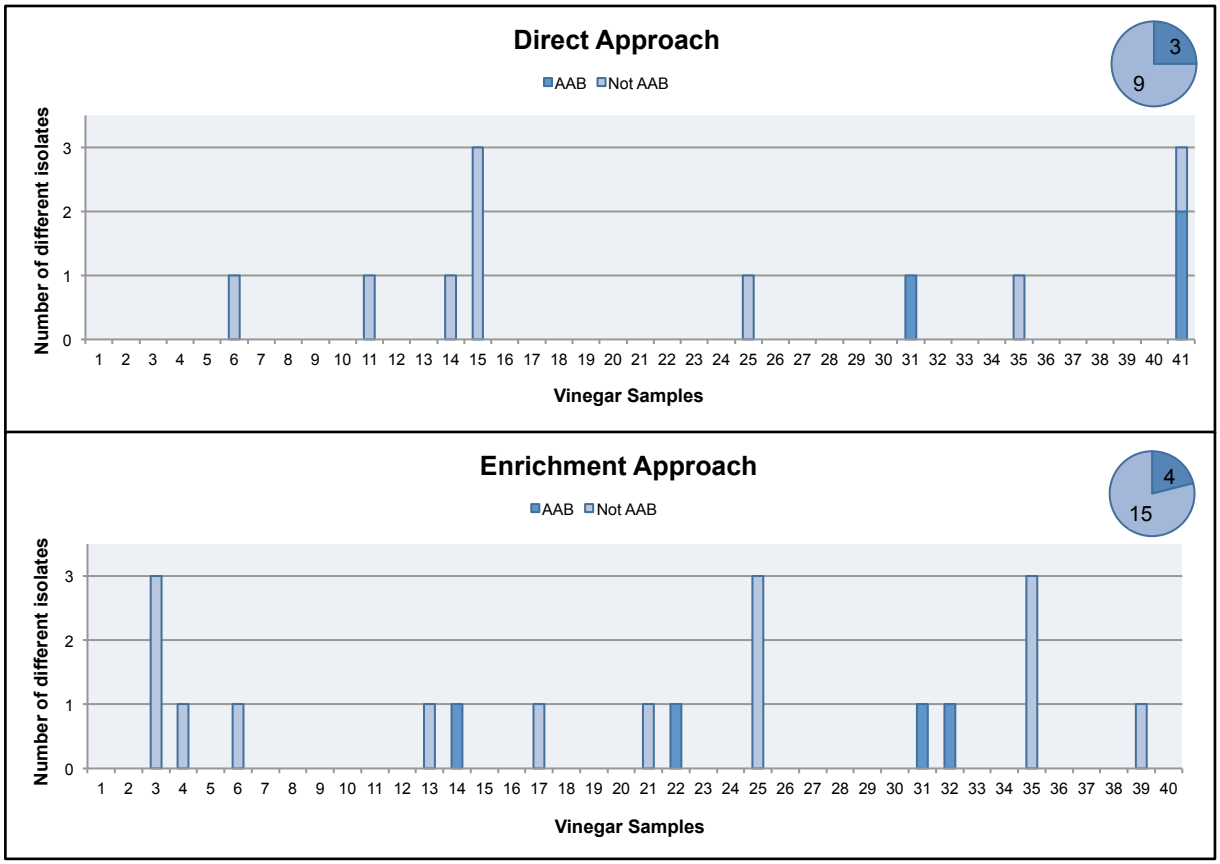


Figure 5. Number of different isolates obtained from each vinegar sample, with the direct and enrichment approaches. The dark blue represents acetic acid bacteria isolates while the light blue represents bacterial isolates not belonging to the acetic acid bacteria group. The pie charts show the total number of isolates for each approach. Only the direct approach was performed for vinegar sample 41.

The direct approach, which consisted in directly plating each vinegar sample in solid medium (GYC), led to the isolation of bacteria from eight of the forty-one vinegars analyzed, totaling twelve different isolates. Of these twelve bacterial isolates, three belonged to the acetic acid bacteria group (confirmed by 16S rRNA sequencing), isolated from vinegars samples 31 and 41. The enrichment approach, which included an incubation step of five days in liquid medium (GYP) supplemented with 3% (v/v) ethanol, led to the isolation of bacteria from thirteen of the forty vinegars analyzed, totaling nineteen different isolates. Of these nineteen bacterial isolates, four belonged to the acetic acid bacteria group (confirmed by 16S rRNA sequencing), isolated from vinegars samples 14, 22, 31 and 32. Additionally,

vinegar samples 1, 5, 8, 9, 18, 27, 34 and 37 showed growth in the liquid medium during the enrichment phase, although no growth was observed when the liquid medium was centrifuged and plated in solid medium.

Table 4 summarizes information about the acetic acid bacteria isolates used in this study, regarding their local and source of isolation as well as their identification (detailed information described in section 2.4 and 3.2). In total, six isolates were isolated in the M&B BioISI Lab and sixteen isolates were isolated in the MG lab.

All bacterial isolates were subjected to Gram, KOH, catalase and oxidase tests. Every acetic acid bacteria was Gram negative, KOH positive, catalase positive and oxidase negative. Additionally, under the microscope cells appeared as small bacilli and occurred preferentially in pairs or in chains. Based on this characterization, both acetic acid bacteria isolated from the sample 31 were considered identical.

Table 4. List of acetic acid bacteria isolated from samples of vinegar produced and/or processed by MG. The isolates are organized by their source and local of isolation. The identification refers to the results of the 16S rRNA gene sequencing (described in section 2.4 and 3.2). MG: Mendes Gonçalves.

Source	Local of isolation	Isolate Name	Identification
Pear vinegar	MG Lab	AAB 001; 002; 003; 004 AAB 010; 011; 012; 017 AAB 028; 029; 030	*Km. swingsii* or *Km. europaeus*
Pepper vinegar		AAB 015; 016; 025; 026 AAB 027	
Cider vinegar	M&B BioISI Lab	AAB 023; 024	*Km. swingsii* or *Km. europaeus*
Cereal vinegar		AAB 031	
Fig vinegar		AAB 032	
Balsamic vinegar		AAB 033; 034	*Km. nataicola* or *Km. sucrofermentans*

The isolation and cultivation of acetic acid bacteria has always been described as problematic, resulting in an underestimation of acetic acid bacteria diversity when culture dependent methods are applied. This is especially true in the isolation from a high acetic acid level source [Raspor *et al*., 2008]. Additionally, a viable but not cultivable (VBNC) state has been described for acetic acid bacteria, mainly in oxygen privation conditions [Mamlouk *et al*., 2013].

When comparing the forty vinegar samples used in both approaches, it is possible to state that the enrichment approach was more effective in the isolation of acetic acid bacteria than the direct approach, since it led to the isolation of four acetic acid bacteria isolates in comparison to one. It is possible that the aeration from the shaking culture helped the acetic acid bacteria recover from the

VBNC state. It is also possible that the availability of ethanol as a carbon source (the same carbon source as in wine/vinegar) may help the bacteria to adapt to the use of glucose as a carbon source. Another way to improve their growth in the solid medium could be its supplementation with ethanol. Also, the enrichment approach seems to have not been specific to acetic acid bacteria, as it was intended, since the number of non-AAB isolates also increased. A possible way to overcome this could be the removal of glucose from the liquid medium, with ethanol as the only carbon source.

As can be seen in Table 4, a higher number of acetic acid bacteria were isolated in the MG lab. A possible explanation could be the reduced time between sample collection and its processing in the lab that can be achieved in the MG lab. This could reduce the interruption of the aeration and induce less stress to the bacteria.

Overall, the enrichment approach was fairly successful, since it led to the isolation of three acetic acid bacteria isolates that otherwise wouldn't be isolated (samples 14, 22 and 32). Still, the analysis of such a high number and diversity of samples was expected to yield a higher number of acetic acid bacteria isolates.

3.2 Typing and Identification of Acetic Acid Bacteria Isolates

A dendrogram-based identification using type strains was applied to twenty-two acetic acid bacteria isolates, purified from vinegar samples, and four reference strains belonging to the genus *Komagataeibacter*. These strains were grouped based on REP-PCR and RAPD-PCR genomic profiles. Figure 6 shows the dendrogram constructed with the Pearson correlation coefficient as a similarity measure and the unweighted pair group method with the arithmetic average clustering algorithm (UPGMA). The reproducibility analysis established a discrimination threshold (97%) below which patterns were deemed different, indicated by the red dotted line. The dendrograms used to determine the reproducibility cutoff are shown in annex A2.

The ERIC-PCR fingerprinting profiles were not used in the construction of the dendrogram because the gel electrophoresis showed substantial differences in the intensity of the amplified bands, especially in the genomic profiles of the reference strains. Therefore, it was decided to exclude this type of genomic fingerprint from the construction of the dendrogram since it was thought that it would introduce distortion. Nevertheless, these fingerprinting profiles are shown here for most acetic acid bacteria isolates as another confirmation of their similarity.

The dendrogram divided the twenty-two acetic acid bacteria isolates into five distinct strains, with strain 1 having the isolates AAB 001, 002, 003, 004, 010, 011, 012, 015, 016, 017, 025, 026, 029 and 030; strain 2 having the isolates AAB 027 and 028; strain 3 having the isolates AAB 023, 024, 031 and 032; and lastly, strains 4 and 5, having the isolates AAB 034 and 033, respectively. Strains 1 and 2 seem to be very closely related to each other, since the only difference observed in the three genomic profiling types is the absence of one band in RAPD-PCR fingerprint. Even though the isolates of strain 3 may seem divided into two different strains of the same species, the differences seen when looking at the banding patterns are mostly resulting from differential amplification of the same bands,

especially in the RAPD-PCR fingerprints. Additionally, the ERIC-PCR profiles also confirm the resemblance of these four isolates. Therefore, the isolates AAB 023, 024, 031 and 032 are considered to be the same strain.

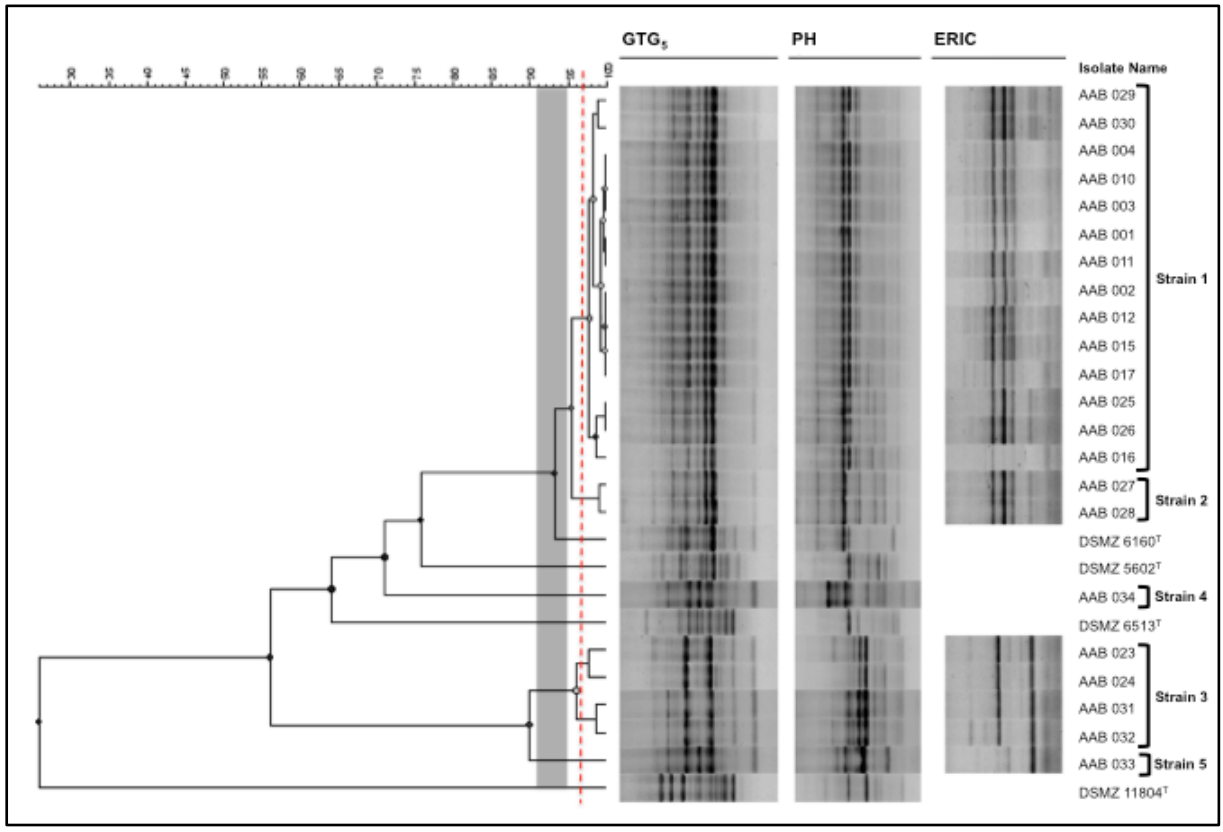


Figure 6. REP-PCR (GTG_5) and RAPD-PCR (PH) fingerprinting patterns from acetic acid bacteria isolates and four reference strains belonging to the genus *Komagataeibacter*. The dendrogram was constructed using the Pearson correlation coefficient as a similarity measure and the unweighted pair group method with the arithmetic average clustering algorithm (UPGMA). The red dotted line represents the cutoff level (97%) determined by the reproducibility analysis and the grey bar shows the cutoff level for species separation. The ERIC-PCR fingerprinting patterns were not used in the construction of the dendrogram. DSMZ 5602: *Km. hansenii*; DSMZ 6160: *Km. europaeus*; DSMZ 6513: *Km. xylinus*; DSMZ 11804: *Km. intermedius*.

The grey bar shown in the dendrogram represents the cutoff level for species separation. The range of similarity between the two different species more closely related (strain 3 and 5) determines this delimitation. Thus, depending on the level of similarity chosen for the cutoff level for species separation, strains 1 and 2 may or may not belong to *Km. europaeus*. Still, these strains show a high level of similarity (93% similarity) with this species. Concerning the isolates of strain 3, they do not show any meaningful similarity with any of the reference strains. The same happens with isolates AAB 033 and 034 (strains 5 and 4, respectively). Therefore, this analysis is not capable of identifying these strains.

Isolates belonging to strains 1 and 2 were all isolated in the MG laboratory. It is interesting to note that they cluster very closely together (95% similarity). Indeed, these isolates were all collected from a 6-liter experimental acetator that is constantly working with different raw materials. This suggests that these isolates may in fact represent two variants of an original resident strain in this experimental acetator, with a notable metabolic plasticity being capable of producing vinegar from at least fermented pear and pepper mashes. Regarding the isolates of strain 3, it is notable to find them grouping together, since they were isolated from cider, cereal and fig vinegar. This could also mean the existence of a resident strain, in an industrial acetator, where these vinegars were produced.

Highly conserved repetitive DNA motifs are distributed throughout the genomes of various bacterial groups, such as Repetitive Extragenic Palindromic (REP) elements or Enterobacterial Repetitive Intragenic Consensus (ERIC) elements. The amplification of the DNA segments between these repetitive sequences has been used to generate fingerprints in several bacterial groups. Additionally, GTG_5-REP-PCR was reported to be a useful fingerprinting technique for identification and classification of acetic acid bacteria to the species level [De Vuyst *et al*., 2008]. Since then, this methodology as been successfully applied in the typification of isolates of this group of bacteria several times [Cleenwerck *et al*., 2010; Vegas *et al*., 2010; Yetiman *et al*., 2015]. The RAPD-PCR methodology with the primer PH has been reported as providing suitable fingerprints, with well defined amplification patterns, appropriate for the identification of *Listeria* spp. [Chambel *et al*., 2007]. As far as we know, it was never applied to acetic acid bacteria. What is interesting about this technique is the primer choice, since it is a universal 16S rRNA gene oriented primer. The amplification reaction is performed with a relatively low annealing temperature and a high concentration of $MgCl_2$ to promote unspecific primer hybridizations, but the primer should always hybridize at its original target. However, to truly unveil the discriminatory power of the RAPD-PCR with the primer PH when applied to acetic acid bacteria, several other type strains would have to be tested, along with a larger collection of isolates. Still, this methodology seems to be promising for the discrimination and classification of this group of bacteria, since it is able to distinguish strain 1 from strain 2 and strain 4 from strain 5, contrarily to GTG_5-REP-PCR.

The sequences obtained with the amplification and sequencing of a portion of the 16S rRNA gene of bacterial isolates were used in a BLAST analysis in search of their closest known relative. However, this analysis was inconclusive since the identification to the species level was not possible, for any of the isolates. Indeed, all of the sequences showed coverage and identity percentages of 99% or more with database sequences belonging to several species of *Komagataeibacter*, the most common being *Km. europaeus*, *Km. xylinus*, *Km. nataicola* and *Km. swingsii* (data not shown). In the light of this result, the sequences obtained with the 16S rRNA gene amplification and sequencing were used in a phylogeny reconstruction (neighbor-joining algorithm), along with the 16S rRNA gene sequences of the type strains of all the species of the genus *Komagataeibacter*, collected from the GenBank database (Figure 7).

The phylogenetic tree shown in Figure 7 cannot distinguish between the isolates AAB 016, 025, 026 and 030 (representatives of strain 1), AAB 027 and 028 (strain 2), AAB 023, 024 and 032 (strain 3)

and the type strains of *Km. europaeus*^{T} and *Km. swingsii*^{T}. This result is in agreement with the results obtained with the genomic fingerprints, where isolates from strains 1 and 2 were shown to be closely related to each other and with *Km. europaeus*^{T}. However, the type strain of *Km. swingsii*^{T} was not used in this study, since it is usually isolated from fruits and fruit juices, resulting in its absence from the genomic fingerprinting analysis. Additionally, on the contrary to the BLAST analysis, the phylogeny shows the distinction between these isolates and *Km. xylinus*^{T} and *Km. nataicola*^{T}.

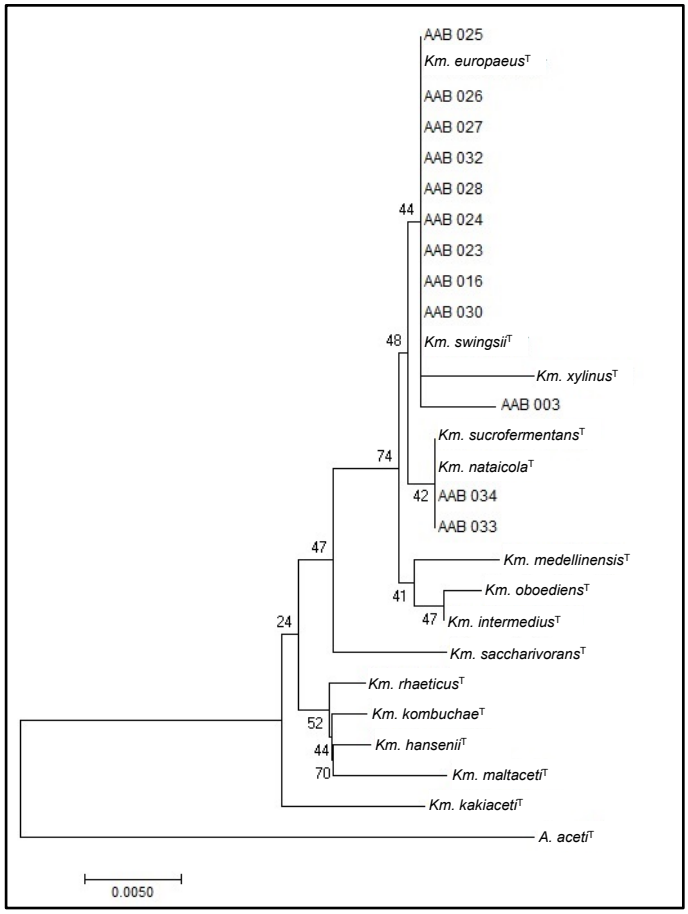


Figure 7. Phylogenetic relationships amongst all species of the genus *Komagataeibacter* and acetic acid bacteria isolates. The phylogenetic tree is based on the sequences of the 16S rRNA gene. Sequences of this gene belonging to the type strains were collected from the GenBank database while the sequences belonging to bacterial isolates were amplified and sequenced, as described in section 2.4. The tree was constructed with neighbor-joining algorithm and numbers at the nodes indicate bootstrap values (%) derived from 1000 replications. *Acetobacter aceti* was used as an outgroup.

Regarding the isolates of strain 3, it was surprising to see how they align with the isolates from strains 1 and 2. This result is in clear contradiction with the difference demonstrated by the analysis of the genomic fingerprints (Figure 6), where all three genomic fingerprinting types noticeably distinguish isolates of strain 3 from those of strains 1 and 2. Additionally, the isolates AAB 033 (strain 5), AAB 034 (strain 4) also align with each other and with the type strains of *Km. nataicola*^{T} and *Km. sucrofermentans*^{T}. Again, this result does not correspond to the interpretation of the dendrogram in Figure 6, where strain 4 and 5 are clearly separated from each other, belonging to different species.

However, the type strains of *Km. nataicola*^{T} and *Km. sucrofermentans*^{T} were not used in the construction of the dendrogram and, since they did not cluster with any of the type strains used, the identification to the species level of the strains 4 and 5 is not possible by this analysis. A possible explanation for this finding is the fact that acetic acid bacteria exhibit highly conserved 16S rRNA gene sequences, which regularly results in an unreliable identification when this marker is used alone [Chakravorty *et al*., 2005]. For this reason, other molecular markers have been explored when working with acetic acid bacteria, such as 16S-23S rRNA ITS or protein-encoding genes (for instance, *rpoB* and/or *dnaK*) [Cleenwerck *et al*., 2010; Trček *et al*., 2005; Trček *et al*., 2002].

The differentiation shown here for the isolate AAB 003 was not expected. Upon further examination of the sequence of the 16S rRNA gene obtained for this isolate, four insertions of adenine were found throughout the sequence. These insertions were not detected in any other sequence belonging to the remaining bacterial isolates. Additionally, none of the results above suggest a reason to differentiate AAB 003 from the remaining isolates of strain 1. Therefore, there is a strong possibility that these insertions are mistakes either in the amplification reaction or in the sequencing process.

Overall, there is a strong suggestion that strains 1 and 2 belong to *Km. europaeus*. Still, the possibility of them belonging to *Km. swingsii* cannot be discarded. Interestingly, the two strains show phenotypic traits both agreeing and disagreeing with those described for these species. Strains belonging to *Km. europaeus* are described as having an absolute requirement of acetic acid for growth [Sievers *et al*., 1992]. However, strains 1 and 2 were always grown on GYC plates, where no acetic acid is added. In fact, such characteristic has already been described for the type strain of *Km. europaeus*, which showed delayed growth in solid RAE medium without acetic acid [Sokollek *et al*., 1998]. With respect to *Km. swingsii* strains, they are commonly isolated from fruits and fruit juices, but not vinegars [Dellaglio *et al*., 2005]. Indeed, strains 1 and 2 were isolated from fermented pear mashes. Another characteristic associated with *Km. swingsii* is cellulose production [Dellaglio *et al*., 2005], although none of these isolates have shown this trait. Regarding strains 4 and 5, they both show cellulose production, which is a characteristic shared with *Km. nataicola* and *Km. sucrofermentans*. Phenotypically, *Km. nataicola* and *Km. sucrofermentans* can be distinguished from each other based on growth on sucrose, where the former is not capable of this and the latter is. Concerning strain 3, the results of both analyses are incoherent since the dendrogram clearly separates this strain from strains 1 and 2 and the phylogeny reconstruction does not distinguish these three strains. Thus, the identification to the species level of any of the strains remains to be confirmed.

3.3 Development of a Molecular Detection Method for Acetic Acid Bacteria

Different amplification conditions and primer combinations were tested in order to optimize the PCR amplification with the designed primers. Table 5 shows the sequential approach to the optimization of the amplification reaction, resulting in the PCR described in section 2.5.

The Multiplex-PCR approach was chosen as a way of increasing the specificity of the methodology, since none of the four possible combinations of primers showed flawless results with the inclusivity and exclusivity controls.

Table 5. Sequential approach to the optimization of the amplification reaction with the designed primers (ADH-F_1, ADH-F_2, ADH-R_1 and ADH-R_2). Inclusivity control refers to strains where amplification is expected to occur. Exclusivity control refers to strains where amplification is not expected to occur. Negative control was performed without adding DNA to the reaction mix.

Tested Conditions	Primers	Tested Strains	Results
Singleplex-PCR ▪ 2 **concentrations of $MgCl_2$** (1.5 mM and 2.5 mM) ▪ 2 **concentrations of primer** (25 pmol and 50 pmol) ▪ a range **of 12 annealing temperatures** (46.5ºC to 57.5ºC)	**F_1R_1** **F_1R_2** **F_2R_2** **F_2R_1**	▪ **Inclusivity control:** *Km. europaeus* DSMZ 6160^T ▪ **Exclusivity control:** *E. coli* ATCC 29522	▪ Amplification of the expected fragment in all conditions; ▪ Amplification in all exclusivity controls with highest $MgCl_2$ concentration; ▪ Selected conditions: 1.5 mM of $MgCl_2$, 25 pmol of primer and annealing temperature at 57ºC;
Singleplex-PCR Amplification in 8 additional inclusivity controls, 1 additional exclusivity control and addition of a negative control	**F_1R_1** **F_1R_2** **F_2R_2** **F_2R_1**	▪ **Inclusivity control:** all reference strains ▪ **Negative control:** no DNA added ▪ **Exclusivity control:** *E. coli* ATCC 29522 and a wild strain of *Morganella morganii*	▪ Amplification of the expected fragment for most inclusivity controls; ▪ Amplification of unspecific bands, mainly for *Acetobacter* strains, when primer R1 was used; ▪ *M. morganii* showed amplification when primer R1 was present; ▪ Negative control showed no amplification;
Multiplex-PCR Amplification with the four primers in the same reaction	**$F_1F_2R_1R_2$**	▪ **Inclusivity control:** all reference strains ▪ **Negative control:** no DNA added ▪ **Exclusivity control:** *E. coli* ATCC 29522 and a wild strain of *M. morganii*	▪ Amplification of the expected fragment for most inclusivity controls; ▪ Amplification of unspecific bands for all inclusivity controls, as well as for both exclusivity controls; ▪ Negative control showed no amplification;
Multiplex-PCR Elimination of primer R_1 and addition of 2 AAB isolates as inclusivity controls and 7 non-AAB strains isolated from vinegar samples as exclusivity controls	**$F_1F_2R_2$**	▪ **Inclusivity control:** all reference strains and 2 AAB isolates ▪ **Negative control:** no DNA added ▪ **Exclusivity control:** *E. coli* ATCC 29522 and 8 non-AAB wild strains	▪ Amplification of the expected fragment for most inclusivity controls, although there is still amplification of one unspecific fragment; ▪ *Acetobacter cerevisiae* CECT 824 shows no amplification; ▪ All exclusivity controls show no amplification;
Multiplex-PCR Addition of an internal (+) control	**$F_1F_2R_2$** and 16S rRNA gene oriented primers **(PA and 907r)**	▪ **Inclusivity control:** all reference strains and 2 AAB isolates ▪ **Negative control:** no DNA added ▪ **Exclusivity control:** *E. coli* ATCC 29522 and 8 non-AAB wild strains	▪ Inclusivity controls show amplification of the internal (+) control, in addition of the expected fragments; ▪ *Acetobacter cerevisiae* CECT 824 only shows amplification of the internal control; ▪ Exclusivity controls show only amplification of the internal control;

Figure 8 shows the Multiplex-PCR profile of an acetic acid bacterium, as well as of bacterial isolates not belonging to this group. The amplification reaction was performed with the optimized conditions. The PCR with the primers ADH-F1, ADH-F2 and ADH-R2 results in the amplification of two fragments in acetic acid bacteria, 240 bp (primers ADH-F2 and ADH-R2), 336 bp (primers ADH-F1 and ADH-R2). Sometimes, the unspecific amplification of a third fragment occurs.

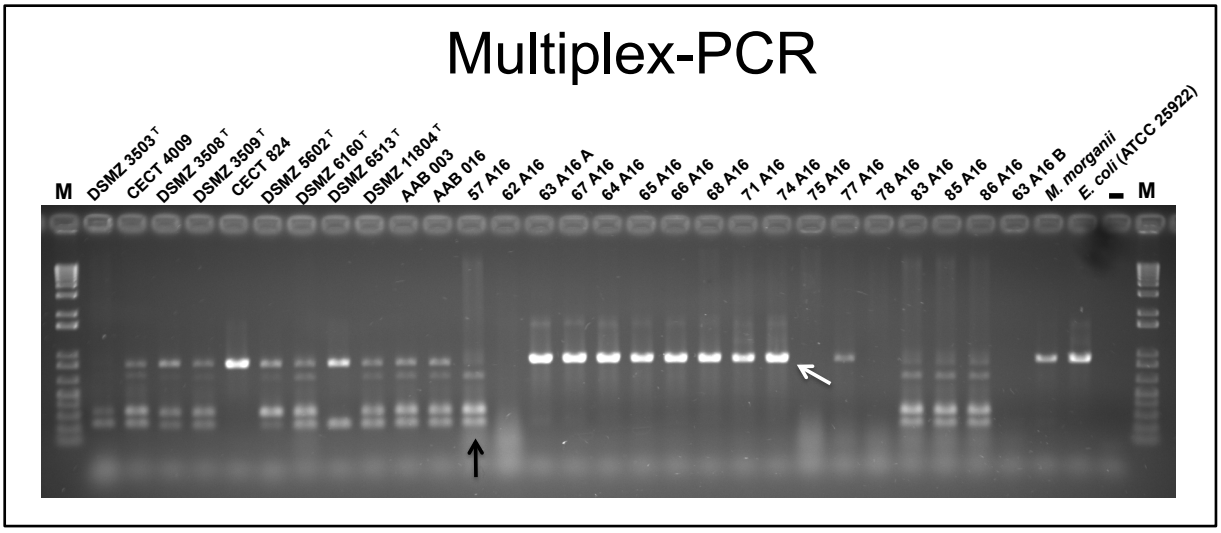


Figure 8. Typical Multiplex-PCR profile of acetic acid bacteria, as well as of bacterial isolates not belonging to this group. The black arrow shows the expected amplification bands for an acetic acid bacteria isolate. The white arrow shows the band corresponding to the internal control (primers PA and 907r). *M. morganii* and *E. coli* ATCC 25922 were used as exclusivity controls, where amplification with the *adhA* directed primers was not expected to occur. The (—) indicates the negative control, where no DNA was added to the mix. The (M) refers to 1kb Plus DNA ladder (Invitrogen).

Here, the Multiplex-PCR amplification reaction is shown, in an electrophoretic separation in agarose gel (1.2%), for all the reference strains used in this study and for two isolates already confirmed to be acetic acid bacteria (AAB 003, 016; *Komagataeibacter* sp.) by 16S rRNA sequencing (chapters 2.4 and 3.2). Additionally, seventeen isolates brought to Lab Bugworkers | M&B-BioISI from the MG lab as potential acetic acid bacteria were also screened. Lastly, *M. morganii* and *E. coli* ATCC 25922 are shown here as exclusivity controls, where amplification with the *adhA* primers was not expected to occur and a negative control (—) where no DNA was added to the PCR mix and no amplification was expected to occur with any of the five primers.

Regarding the reference acetic acid bacteria, two of the eleven strains do not show the expected amplification profile. In *Km. xylinus* DSMZ 6513^{T}, only the smallest fragment can be seen, corresponding to the 240 bp fragment (primers ADH-F2 and ADH-R2). Also, *A. cerevisiae* CECT 824, showed a negative result. Concerning the seventeen unidentified bacterial isolates, four show the profile corresponding to acetic acid bacteria, nine exclusively show the amplification of the internal control (negative result) and four do not show any band at all (possibly a problem in the DNA extraction). Finally, the exclusivity controls demonstrate the negative result of the amplification

reaction. Thus, Figure 8 shows how clearly acetic acid bacteria can be distinguished from of non-AAB isolates.

In any industrial process, the identification and quantification of different strains and species is extremely important. Since acetic acid bacteria are involved not only in the production, but also in the spoilage of foods and beverages, the monitoring of their presence is essential in the different stages of an industrial bioprocess, especially in the final product, after the manufacturing process [Raspor *et al*., 2008]. Since identification methodologies based on phenotypic characteristics of acetic acid bacteria are not only unreliable, but also time-consuming, the application of molecular detection and/or identification methods could provide a fast and accurate solution [Trček *et al*., 2005]. Additionally, molecular methods are the only option when working with microorganisms of difficult isolation or in a VBNC state.

The discrimination power of a molecular detection method is extremely dependent on its molecular target. The 16S rRNA gene of acetic acid bacteria is very conserved and as been shown to have different hypervariable regions than those normally described for other bacterial groups [Chakravorty *et al*., 2005]. Therefore, this gene was initially assessed as a possible target for the development of a molecular detection method specific for acetic acid bacteria. However, since it is present in all bacteria, a gene with a more conserved function was thought to be more indicated for the desired methodology. The unique ability of this group of bacteria to oxidize ethanol to acetic acid is due to two periplasmic proteins, PQQ-dependent ADH and ALDH [Matsushita *et al*., 2016]. Therefore, the gene *adhA*, which encodes for the subunit I of the PQQ-ADH was evaluated as a potential molecular target. A multiple alignment of sequences of this gene belonging to different acetic acid bacteria species showed the presence of variable and conserved segments, ideal for the design of oligonucleotide primers.

The gene *adhA* has already been studied and was shown to be more and less discriminatory for acetic acid bacteria when compared with the 16S rRNA gene and the 16S-23S rRNA ITS, respectively. Also, *A. aceti* specific primers were designed and the gene *adhA* was reported to be a promising target for the construction of species-specific oligonucleotides for quick molecular identification of acetic acid bacteria [Trček *et al*., 2005].

In the present study, the designed primers were shown to be specific for acetic acid bacteria, since all the non-AAB isolates tested were negative (only showed amplification of the internal control). Besides, all these non-AAB strains were isolated from vinegar samples. The 16S rRNA gene was amplified and sequenced (as described in section 2.4) and these isolates were identified as *Staphylococcus* spp., *Bacillus* spp. and *Paenibacillus* spp.. Still, one of the reference strains, *A. cerevisiae* CECT 824, showed a negative result, meaning that the primer ADH-R2 does not hybridize in the *adhA* sequence of this strain and that the designed primers were not 100% inclusive in the tested conditions. Additionally, *Km. xylinus* DSMZ 6513^T shows amplification of only one band, instead of the expected two, suggesting that ADH-F_1 does not hybridize in the *adhA* sequence of this strain. During the development of the amplification reaction (Table 5), problems were also detected with the primer ADH-R1 in the strains *A. aceti* DSMZ 3508^T and *A. pasteurianus* DSMZ 3509^T. However, these

complications can also be seen as opportunities. If the primer does not hybridize in a certain type strain, sequence differences may exist to open the road for the use of *adhA* gene to build species-specific probes.

Taking everything into account, these results show that the designed primers, and the optimized amplification reaction, are effective in the molecular detection of acetic acid bacteria and that once again, the gene *adhA* has been successfully used for this purpose, even though different regions were explored. This methodology was applied as a routine detection method to several unidentified bacterial isolates (additional form those shown in Figure 8) brought to the Lab Bugworkers | M&B-BioISI from the MG lab and it proved to be a fast and reliable methodology in the distinction of acetic acid bacteria from non-AAB isolates.

3.4 Growth Performance of Acetic Acid Bacteria in Red Wine

Acetic acid bacteria isolates AAB 023, 030, 033 and 034, corresponding to strains 3, 1, 5 and 4, respectively, were inoculated in diluted wine (around 4.8% ethanol concentration) and their growth was followed for several days. Figure 9 shows the growth characteristics of these four strains, as well as the pH of the medium and the concentration of acetic acid throughout several days. All strains were grown in triplicates and all optical density (OD), pH and acetic acid concentration measurements were obtained for each triplicate.

Table 6 shows the main growth characteristics of these four strains. The isolate that showed the shortest lag phase was AAB 030, around one day, while isolates AAB 023 and 033 showed a lag phase of around two days and AAB 034 showed lag phase of almost four days. Regarding the cell yield, isolates AAB 023 and 030 showed the highest values, reaching OD values of 0.9 and 0.75, respectively, and the highest growth rates, 0.28 day^{-1} and 0.34 day^{-1}, respectively, while isolates AAB 033 and 034 showed very low cell yield values and growth rates. With the exception of isolate AAB 033, all isolates showed an abrupt decrease in cell number after the highest acetic acid concentration was reached. The highest acetic acid concentration, around 4% (w/v), was obtained with isolate AAB 023, while around 3.2% (w/v) acetic acid was obtained with isolates AAB 030 and 033 and around 2.8% (w/v) acetic acid was obtained with isolate AAB 034. Interestingly, isolates AAB 033 and 034 showed the highest acetic acid production rates, 0.37 $\%day^{-1}$ and 0.38 $\%day^{-1}$, respectively.

In general, these four isolates show two distinct growth types that seem to be associated to the production of cellulose. Isolates AAB 033 and 034 are cellulose producers. This is quite possibly the explanation for the low cell yield measured, since cells of these strains grow primarily on the cellulose matrix. When a sample of the culture was collected to measure the OD, agglomerates of cellulose were either too big and did not enter the pipette tip or small enough to enter the pipette tip but interfered with the absorbance reading. Interestingly, these strains showed different behaviors in terms of cellulose production. At the time of the collection of the last time-point (day 28), all triplicates of AAB 033 showed turbidity in the medium, along with several chunks of cellulose, while all triplicates of AAB 034 showed only one big mass of cellulose and the medium was completely clear (as evidenced

by the OD reading). Strains that produce cellulose are not indicated in the production of vinegar through the submerged fermentation process since the cellulose produced may clog the tubing.

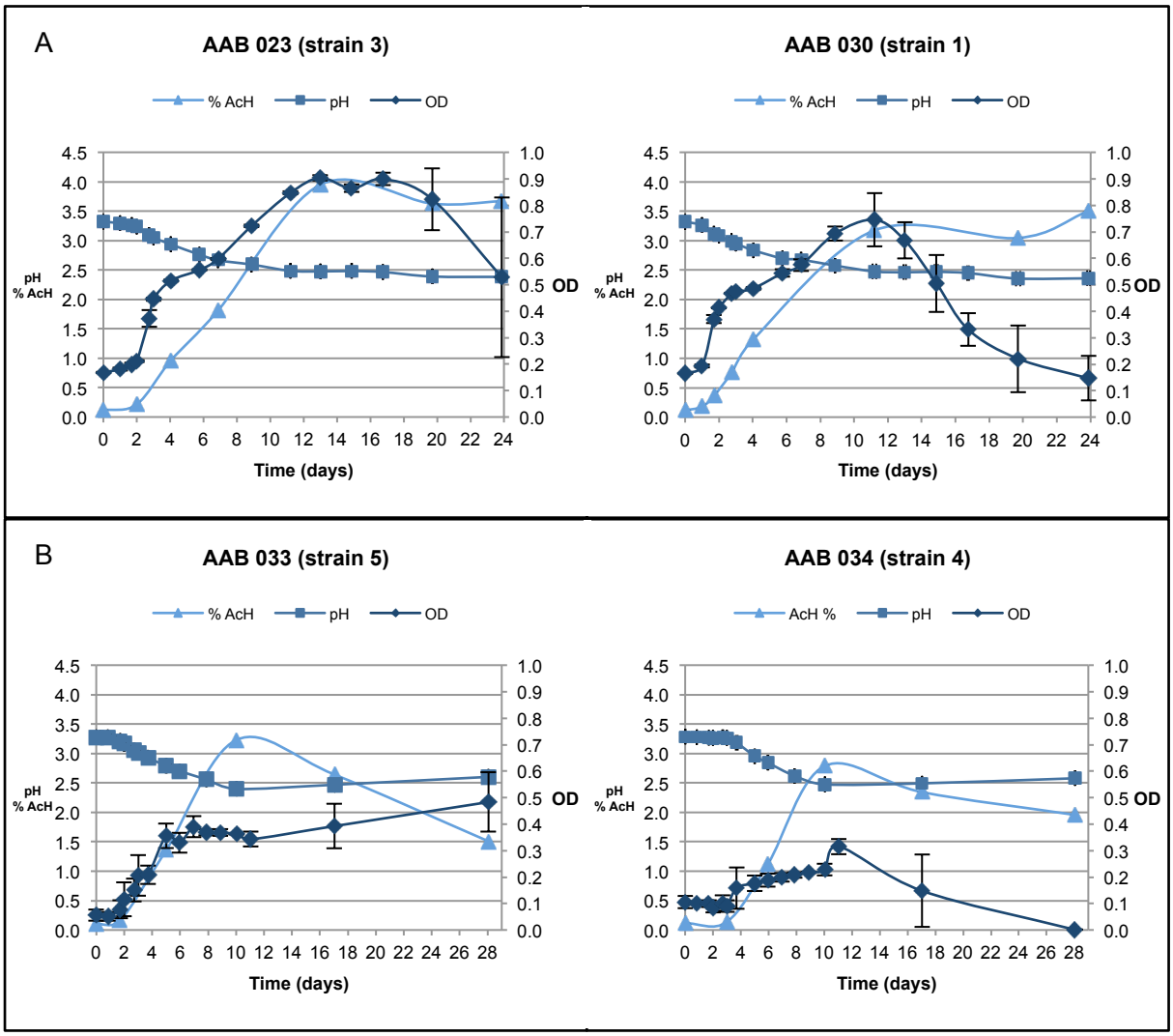


Figure 9. Growth characteristics of strains of acetic acid bacteria in diluted wine. A: non-cellulose producing isolates AAB 023, 030, corresponding to strains 3, 1, respectively. B: cellulose producing isolates AAB 033 and 034, corresponding to strains 5 and 4, respectively. All strains were grown in triplicates, at 28ºC and shaking conditions. Error bars represent the standard deviation at each time-point. OD: optical density measured at 600 nm (◆); pH (■); % AcH: acetic acid concentration (w/v) (▲).

The maximal bioconversion efficiency was calculated with the assumption that the initial ethanol concentration was 4.8% (v/v) and that it was completely consumed by all strains. Isolate AAB 023 showed the highest bioconversion efficiency, 78.3%, and, as expected, isolates AAB 033 and 034 showed lower values, 64.2% and 55.2%, respectively, since they must redirect carbon and energy for the production of cellulose. The low bioconversion efficiency shown by isolate AAB 030 was not expected since there is no carbon draining for cellulose production. It is possible that this strain has a low resistance to acetic acid. That would explain the low maximum acetic acid concentration reached and the low bioconversion efficiency (if not all ethanol was consumed). Regarding productivity, this

parameter differs from acetic acid production rate because it takes in account the time necessary to reach the maximum acetic acid concentration. As expected, isolate AAB 023 shows a high productivity, 2.96 $gl^{-1}day^{-1}$. Still, isolate AAB 033 achieved the highest productivity, 3.11 $gl^{-1}day^{-1}$. This could be explained by the fact that isolate AAB 033 reached the maximum acetic acid concentration in 10 days while isolate AAB 023 reached the maximum acetic acid concentration in 13 days.

Table 6. Growth parameters of isolates AAB 023, 030, 033 and 034, equivalent to strains 3, 1, 5 and 4, respectively.

	AAB 023	AAB 030	AAB 033	AAB 034
Lag phase (hours)	41	24	48	89
Growth rate 1st exponential (day^{-1}) [a]	0.28	0.34	0.10	0.02
Growth rate 2nd exponential (day^{-1}) [a]	0.03	0.03	0.01	not applicable
Maximum OD	0.90	0.75	0.39	0.32
Acetic acid production rate (%day^{-1}) [b]	0.34	0.29	0.37	0.38
Maximum acetic acid obtained % (w/v)	3.95	3.18	3.23	2.79
Maximal bioconversion Efficiency (%)	78.3	62.9	64.2	55.2
Productivity ($gl^{-1}day^{-1}$)	2.96	2.73	3.11	2.66
Cellulose production	-	-	+	+

[a] Determination coefficient (R^2) varied between 0.888 and 0.998; [b] Determination coefficient (R^2) varied between 0.991 and 0.997.

It is interesting to see that although isolates AAB 023 and 030 show a diauxic growth curve, the second exponential growth phase does not correspond to the assimilation of acetic acid, as is described for *Komagataeibacter* strains. Indeed, these isolates did not seem to consume acetic acid. It has been previously described that overoxidation only occurs in *Km. europaeus* strains when the acetic acid concentration is below a strain-specific limit, varying between 5% and 9%, at the time of ethanol depletion [Sokollek *et al*., 1998]. However, acetic acid concentration did not reach these values with either isolate.

Overall, strains 1 and 3, represented by isolates AAB 030 and 023, respectively, appear to be indicated to be employed as starter cultures since they show absence of ethanol overoxidation and cellulose production. Strain 1 showed a shorter lag phase and higher growth rate, while strain 3 showed high cell yield, acetic acid production rate and final concentration, bioconversion efficiency and productivity. An interesting approach to an arrest of an industrial acetification process would be the employment of a mixed inoculum of these two strains. Strain 1 would be responsible for a fast restart of the acetification, giving time for strain 3 to thrive and take over the vinegar production. Also, strain 5 could be indicated for the production of static culture or balsamic vinegars, since it is a

cellulose producer but, at the same time, this strain shows acceptable values of lag phase, acetic acid production rate and maximum concentration, bioconversion efficiency and the highest productivity. However, additional characterization of these strains is needed to truly determine their suitability as starters.

3.5 Microbial Community of a Red Wine Vinegar Production Cycle

Community DNA was extracted from five red wine vinegar samples collected from the same acetator, throughout 36 hours, corresponding to different stages of a red wine vinegar production cycle. Figures 10 and 11 show the proportion of acetic acid bacteria at each time-point of the vinegar production cycle. These figures refer to the results of the amplification, sequencing and identification of regions 1 and 2 of the 16S rRNA gene, respectively. Here is presented the evolution of the acetic acid bacteria population throughout the conversion of red wine into vinegar.

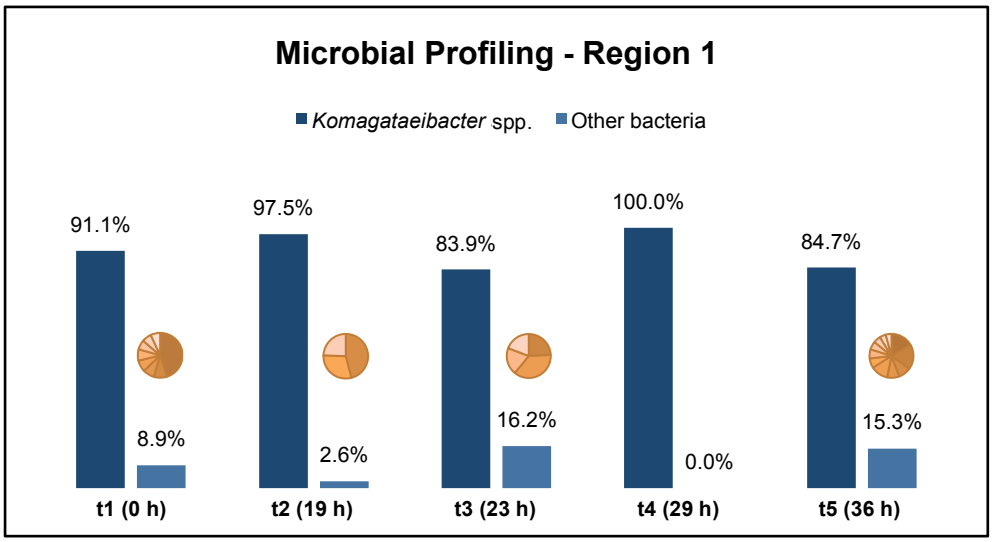


Figure 10. Representation of the proportion of acetic acid bacteria in each time-point. The pie charts show the relative abundance of non-AAB in each time-point. Data is relative to the analysis of the region 1 of the 16S rRNA gene. Percentages were calculated from a total of 39 093 reads.

Detailed information about this analysis is shown in annex A4. Additionally, the presence of fungi was investigated with ITS directed primers, although the results were negative for all samples.

As can be observed in both figures, the production process starts with an elevated proportion of acetic acid bacteria, of around 90% (t1, 0 h). This value progressively increases throughout 29 hours until a total absence of non acetic acid bacteria is reached (t4, 29 h). In time-point 5, a decrease of the proportion of acetic acid bacteria can be seen, corresponding to the end of the cycle, when the acetator is partially emptied and refilled with fresh mash (wine).

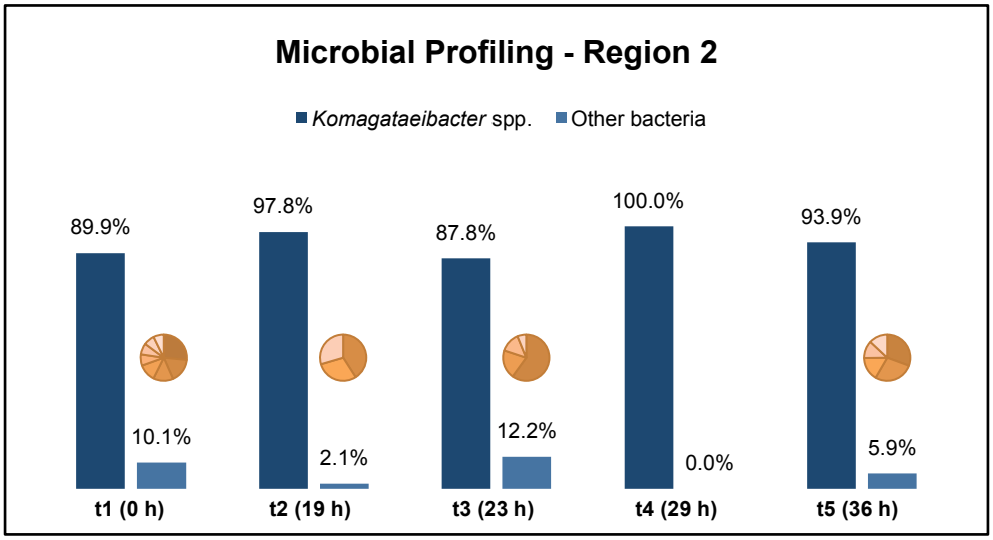


Figure 11. Representation of the proportion of acetic acid bacteria in each time-point. The pie charts show the relative abundance of non-AAB in each time-point. Data is relative to the analysis of the region 2 of the 16S rRNA gene. Percentages were calculated from a total of 45 997 reads.

In time-point 3, a clear disruption of the increase of the proportion of acetic acid bacteria can be seen, in both figures. Since this disturbance seems deranged from the general tendency demonstrated by this analysis, the possibility of contamination with external microorganisms can't be excluded, either at the moment of collection of the sample or in any of its downstream processing. Additionally, no problems with this production cycle were reported by MG. Thus, it is assumed that the data presented in this time-point does not represent the true microbial community in the acetator at that time.

These results show that this process is clearly dominated by acetic acid bacteria, particularly those belonging to the genus *Komagataeibacter*. Here is evidenced how well adapted these organisms are, thriving in such a harsh environment. Additionally, this result is in agreement with other studies that confirm that *Komagataeibacter* strains are indeed the most resistant to acetic acid, within the acetic acid bacteria, and thus are generally responsible for acetification in submerged fermentations, with a high yield of acetic acid production [Sievers *et al*., 1992; Trček *et al*., 2016].

The fact that two different regions of the 16S rRNA gene demonstrate results with such a high level of similarity increases the confidence in the analysis. However, the taxonomic resolution with this type of technique is extremely dependent on the molecular target selected. The rRNA operon has been widely used for studying prokaryotic communities, especially the 16S subunit, as it contains highly conserved regions (ideal for universal primer locations) intercalated by highly heterogenic regions (ideal for taxonomic discrimination) [Bokulich *et al*., 2012]. Still, not all taxa are equally heterogeneous at a given region. As discussed above, acetic acid bacteria are known to have highly conserved 16S rRNA gene sequences [Raspor *et al*., 2008; Trček *et al*., 2016]. Another problem often associated with this molecular marker is its copy number variation and how this variation can distort the results, with bacterial groups being misrepresented. Consequently, protein-encoding genes have been investigated, such as *rpoB* (encoding the *β*-subunit of the RNA polymerase), though these targets are

less studied across all bacterial taxa [Bokulich *et al*., 2012]. Thus, the 16S rRNA gene remains the most prevalent molecular target as it is the most represented prokaryotic gene in public databases.

Earlier this year, NGS (Ilumina) of vinegar samples collected form an industrial acetator was applied for the first time, both in wine and cider vinegar production, targeting the variable region V3-V4 of the 16S rRNA gene [Trček *et al*., 2016]. The authors reported *Komagataeibacter* sp. as the dominant microorganism in red wine vinegar production, with a lowest relative abundance of 84% and a highest of 99%, throughout several time-points of three different production batches. These results are in full agreement with those presented here. However, this is still a pseudo-quantitative method, showing only relative abundance of the detected microorganisms and only one production cycle was analyzed, from an acetator constantly running.

4. Final Remarks and Future Perspectives

In this work, acetic acid bacteria were isolated from a large variety of vinegars and were grouped based on two genomic fingerprinting techniques, GTG_5-REP-PCR and PH-RAPD-PCR, along with reference strains obtained from culture collections. These genomic profiles were used to define 5 different strains and isolates belonging to all strains were randomly selected for the amplification and sequencing of the 16S rRNA gene. A BLAST analysis and a phylogenetic reconstruction positioned all strains in the genus *Komagataeibacter*. However, due to the high level of conservation of this gene in this group of bacteria, the identification to the species level of any of the strains was not possible. Four acetic acid bacteria isolates, belonging to four different strains, were grown in diluted red wine in order to evaluate the suitability of the employment of these strains as starter cultures. Strains 1 and 3 showed desirable characteristics of an optimal acetic acid bacteria starter, such as a short lag phase, high cell yield, high bioconversion efficiency and productivity, no ethanol overoxidation and no cellulose production. Additionally, a molecular detection method for acetic acid bacteria was developed targeting the *adhA* gene. Primers were designed based on a multiple alignment of sequences of this gene belonging to different acetic acid bacteria species and genera. The PCR was optimized and tested on 9 reference strains and a variety of bacterial isolates not belonging to the acetic acid bacteria group. This methodology proved to be fast and reliable in the distinction of acetic acid bacteria from non-AAB isolates. Finally, community DNA was extracted from five vinegar samples, corresponding to different stages of a red wine vinegar production cycle. Two regions of the 16S rRNA gene were amplified, sequenced by NGS and identified by a BLAST analysis. The results showed that the vinegar production through the submerged fermentation process is clearly dominated by acetic acid bacteria, particularly those belonging to the genus *Komagataeibacter*.

In the last years, several media specific for the growth of acetic acid bacteria have been described. It would be interesting to revisit the isolation approaches with the employment of different solid and liquid media for the isolation of acetic acid bacteria, namely the use of RAE solid medium, that has acetic acid and ethanol in the medium composition and a double-layer agar [Sokollek *et al*., 1997]. The double layer is made using a lower layer of medium with 0.5% agar and a top layer of medium with 1% agar. The semi-solid lower layer provides the colonies growing on the top layer a constant supply of moisture, simulating the environment in the acetification tanks. Other possibility is the usage of a liquid medium with ethanol as the only carbon source in the enrichment approach.

Regarding the acetic acid bacteria isolated in this study, it would be interesting to identify them to the species level. Ideally, a molecular methodology targeting other genes than the 16S rRNA gene should be applied. A multilocus sequence analysis (MLSA) with the genes *dnaK* (encoding a heat-shock protein), *rpoB* (encoding the *β*-subunit of the RNA polymerase) and *groEL* (encoding a chaperone protein) proved to be suitable for acetic acid bacteria species differentiation and also led to the reclassification of a strain of *Komagataeibacter xylinus* [Cleenwerck *et al*., 2010].

Strains 1 and 3 were shown to be promising candidates to be employed as starter cultures. However, further tests are necessary to determine their suitability as starters regarding cell viability after frozen and lyophilized preparations, genomic stability and maintenance of phenotype, organoleptic

characteristics and acetic acid production yield and productivity in a vinegar “fermentation” at an industrial scale.

Regarding the multiplex-PCR, the sequence of the *adhA* gene showed to be promising for the design of species-specific probes, since it presented conserved and variable regions. Indeed, this gene was indicated as having higher variability among different species of acetic acid bacteria than the 16S rRNA gene [Trček *et al*., 2005]. In this study, we found differential amplification in different species with the four designed primers. These primer hybridization regions should be further explored since they are promising for the construction of species-specific probes.

5. References

Adler, P., Frey, L.J., Antje Berger, Bolten, C.J., Hansen, C.E., Wittmanna, C., 2014. The Key to Acetate: Metabolic Fluxes of Acetic Acid Bacteria under Cocoa Pulp Fermentation-Simulating Conditions. Applied and Environmental Microbiology, 80, 4702–4716.

Bokulich, N., Mills, D., 2012. Next-generation approaches to the microbial ecology of food fermentations. BMB reports, 45(7): 377-389.

Chakravorty, S., Sarkar, S., Gachhui, R., 2005. Identification of New Conserved and Variable Regions in the 16S rRNA Gene of Acetic Acid Bacteria and Acetobacteraceae Family. Molecular Biology, Vol. 49, No. 5, pp. 668–677.

Chambel, L., Sol M., Fernandes, I., Barbosa, M., Zilhão, I., Barata, B., Jordan, S., Perni, S., Shama, G., Adrião, A., Faleiro, L., Requena, T., Peláez, C., Andrew, P.W., Tenreiro, R., 2007. Occurrence and persistence of *Lysteria* spp. in the environment of ewe and cow's milk dairies in Portugal unveiled by an integrated analysis of identification, typing and spatial-temporal mapping along production cycle. International Journal of Food Microbiology, 116, 52-63.

Cleenwerck, I., De Vos, P., De Vuyst, L., 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. International Journal of Systematic and Evolutionary Microbiology, 60, 2277–2283.

De Vuyst, L., Camu, N., De Winter, T., Vandemeulebroecke, K., Van de Perre, V., Vancanneyt, M., De Vos, P., Cleenwerck, I., 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. International Journal of Food Microbiology 125, 79–90.

Dellaglio, F., Cleenwerck, I., Felis, G.E., Engelbeen, K., Janssens, D., Marzotto, M., 2005. Description of *Gluconacetobacter swingsii* sp. nov. and *Gluconacetobacter rhaeticus* sp. nov., isolated from Italian apple fruit. International Journal of Systematic and Evolutionary Microbiology, 55, 2365–2370.

Deppenmeier, U., Hoffmeister, M., Prust, C., 2002. Biochemistry and Biotechnological Applications of *Gluconobacter* strains. Applied Microbiology Biotechnology, 60:233–242.

Fernández-Pérez, R., Torres, C., Sanz, S., Ruiz-Larrea, F., 2010. Strain typing of acetic acid bacteria responsible for vinegar production by the submerged elaboration method. Food Microbiology 27, 973-978.

Guillamón, J.M., Mas, A., 2009. Acetic Acid Bacteria. *In Biology of Microorganisms on Grapes, in Must and in Wine* (pp. 31-46). König, H., Unden, G., Fröhlich, J. (Eds.) Springer, Heidelberg.

Guillámon, J.M., Mas, A., 2011. Acetic Acid Bactria. *In Molecular Wine Microbiology* (pp. 227-255). Carrascosa, A.V., Muñoz, R., González, R. (Eds.) Elsevier, UK.

Gullo, M., Giudici, P., 2008. Acetic acid bacteria in traditional balsamic vinegar: Phenotypic traits relevant for starter cultures selection. International Journal of Food Microbiology 125, 46–53.

Gullo, M., Verzelloni, E., Canonico, M., 2014. Aerobic submerged fermentation by acetic acid bacteria for vinegar production: Process and biotechnological aspects. Process Biochemistry 49, 1571-1579.

Komagata, K., Iino, T., Yamada, Y., 2014. The Family *Acetobacteraceae*. *In The Prokaryotes*, (pp. 3-78) Rosenberg, E., *et al*. (Eds.) Springer, New York.

Li, L., Praet, J., Borremans, W., Nunes, O.C., Manaia, C.M., Cleenwerck, I., Meeus, I., Smagghe, G., De Vuyst, L., Vandamme, P., 2015. *Bombella intestini* gen. nov., sp. nov., an acetic acid bacterium isolated from bumble bee crop. International Journal of Systematic and Evolutionary Microbiology, 65, 267–273.

Mamlouk, D., Gullo, M., 2013. Acetic Acid Bacteria: Physiology and Carbon Sources Oxidation. Indian Journal of Microbiology, 53:377–384.

Mas, A., Torija, M.J., García-Parrilla, M.C., Troncoso, A.M., 2014. Acetic Acid Bacteria and the Production and Quality of Wine Vinegar. The Scientific World Journal, Article ID 394671, 6 pages.

Massol-Deya, A.A., Odelson, D.A., Hickey, R.F., Tiedje, J.M., 1995. Bacterial community fingerprinting of amplified 16S and 16-23 ribossomal DNA gene sequences and restriction endonuclease analysis (ARDRA). Molecular Microbial Ecology Manual, 3.3.2: 1-8.

Matsushita, K., Toyama, H., Adachi, O., 2004. Diversity of Prokaryotic Respiratory Systems. *In Respiration in Archaea and Bacteria*. (pp. 81-99). Davide Zannoni, (Ed.) Springer, The Netherlands.

Matsushita, K., Toyama, H., Tonouchi, N., Okamoto-Kainuma, A., 2016. *Acetic Acid Bacteria, Ecology and Physiology*. Springer, Japan.

Mounir, M., Shafiei, R., Zarmehrkhorshid, R., Hamouda, A., Thonart, P., Delvigne, F., Alaoui, M.I., 2016. Optimization of biomass production of Acetobacter pasteurianus KU710511 as a potential starter for fruit vinegar production. African Journal of Biotechnology, Vol. 15(27), pp. 1429-1441.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H., Wawer, C., 1998. Denaturating gradient gel electrophoresis (DGGE) in microbial ecology. Molecular Microbial Ecology Manual, 3.4.4: 1-27.

Nanda, K., Taniguchi, M., Ujike, S., Ishihara, N., Mori, H., Ono, H., Murooka, Y., 2001. Characterization of Acetic Acid Bacteria in Traditional Acetic Acid Fermentation of Rice Vinegar (Komesu) and Unpolished Rice Vinegar (Kurosu) Produced in Japan. Applied And Environmental Microbiology, p. 986–990.

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer. A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U., 2005. Complete Genome Sequence of the Acetic Acid Bacterium *Gluconobacter oxydans*. Nature Biotechnology, volume 3, number 2.

Raspor, P., Goranovič, D., 2008. Biotechnological Applications of Acetic Acid Bacteria. Critical Reviews in Biotechnology, 28:101-124.

Sakurai K., Arai H., Ishii, M., Igarashi, Y., 2012. Changes in the gene expression profile of Acetobacter aceti during growth on ethanol. Journal of Bioscience and Bioengineering, Vol. 113 No. 3, 343–348.

Sievers, M., Swings, J., 2005. Family II. Acetobacteraceae. *In Bergey's Manual of Systematic Bacteriology*, 2nd Edition, Volume II, Part C (pp. 41-95). Brenner, D.J., Krieg, N.R., Staley, J.T. (Eds.) Springer, New York.

Sievers, M., Sellmer, S., Teuber, M., 1992. *Acetobacter europaeus* sp. nov., a Main Component of Industrial Vinegar Fermenters in Central Europe. Systematic and Applied Microbiology, 15, 386-392.

Sokollek, S.J., Hammes, W.P., 1997. Description of a Starter Culture Preparation for Vinegar Fermentation. Systematic and Applied Microbiology, 20: 481–491.

Sokollek, S.J., Hertel, C., Hammes, W.P., 1998. Cultivation and Preservation of Vinegar Bacteria. Journal of Biotechnology, 60, 195-206.

Trček, J., 2005. Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S–23S rDNA internal transcribed spacer region and of the PQQ-dependent alcohol dehydrogenase gene. Systematic and Applied Microbiology 28, 735–745.

Trček, J., Mahnič, A., Rupnik, M., 2016. Diversity of the microbiota involved in wine and organic apple cider submerged vinegar production as revealed by DHPLC analysis and next-generation sequencing. International Journal of Food Microbiology, 223, 57–62.

Trček, J., Teuber, M., 2002. Genetic and restriction analysis of the 16S-23S rDNA internal transcribed spacer regions of the acetic acid bacteria. FEMS Microbiology Letters, 208, 69-75.

Vegas, C., Mateo, E., González, Á., Jara, C., Guillamón, J.M., Poblet, M., Torija, M.J., Mas, A., 2010. Population dynamics of acetic acid bacteria during traditional wine vinegar production. International Journal of Food Microbiology 138, 130–136.

Versalvoice, J., Kouetch, T., Lupski, J.R., 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genome. Nucleic Acids Research, 19: 6823– 6831.

Yamada, Y., Yukphan, P., Vu, H.T.L., Muramatsu, Y., Ochaikul, D., Tanasupawat, S., and Nakagawa, Y., 2012. Description of Komagataeibacter gen. nov., with proposals of new combinations (Acetobacteraceae). Journal of General and Applied Microbiology, 58, 397–404.

Yetiman, A.E., Kesmen, Z., 2015. Identification of acetic acid bacteria in traditionally produced vinegar and mother of vinegar by using different molecular techniques. International Journal of Food Microbiology, 204, 9–16.

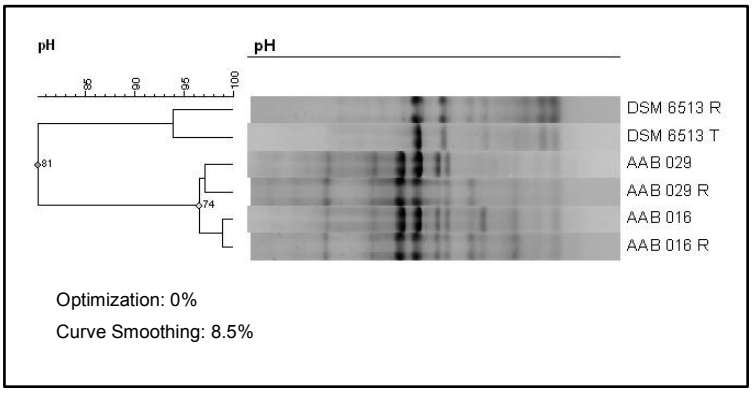
Annexes

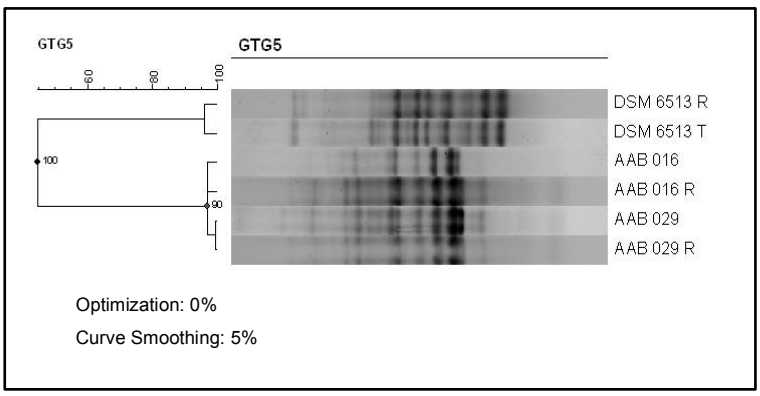
A1. Composition of culture media

A medium that allows the success isolation of acetic acid bacteria from different niches is Glucose Yeast-extract Calcium Carbonate Agar (GYC), composed by glucose (5%), yeast extract (0.5%), peptone (0.3%), calcium carbonate ($CaCO_3$) (2%) and agar (1.5%). After incubation, the colonies of acetic acid bacteria are recognized by the surrounding zones of calcium carbonate clearing. The calcium carbonate reacts with the produced acid, neutralizing it and preventing physiological stress and cell death [Mamlouk *et al*., 2013]. Glucose Yeast-extract Peptone (GYP) has the same composition of as the GYC medium, with the exception of calcium carbonate and the agar.

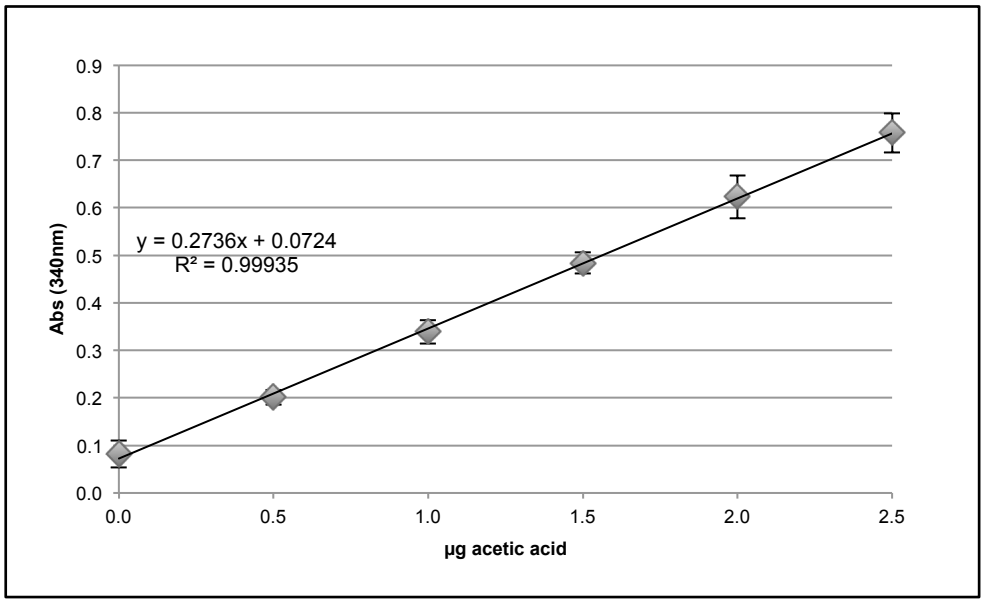
Reinforced Acetic Acid-Ethanol (RAE) medium is composed of glucose (4%), yeast extract (1%), peptone (1%), disodium hydrogen phosphate dihydrate ($Na_2HPO.2H_2O$) (0.338%) and citric acid (0.15%) (w/v). The solid medium has a double-layer agar [Sokollek *et al*., 1997]. The double layer is made using a lower layer of medium with 0.5% agar and a top layer of medium with 1% agar. The semi-solid lower layer provides the colonies growing on the top layer a constant supply of moisture, simulating the environment in the acetification tanks. Acetic acid and ethanol are added to the medium composition and their final concentrations are indicated as follows: RAE (1a/2e) means that 1 ml of glacial acetic acid and 2 ml of absolute ethanol are added per 100 ml of medium.

A2. Dendrograms used to determine the reproducibility cutoff





A3. Calibration curve estimated for the enzymatic quantification of acetic acid. The data is relative to three replications.



A4. NGS results. 16S rRNA gene region 1:

Sample	Species	Results in		% coverage	% identity
		(%)	(nº seqs)		
A1 (t0, 0h)	*Komagataeibacter* spp.	91.14	751	95	100
	Acinetobacter spp.	4.00	33	99	100
	Pseudomonas spp.	0.85	7	99	98
	Sphingomonas spp.	0.73	6	99	99
	Oenococcus oeni	0.73	6	99	100
	Undibacterium oligocarboniphilum	0.73	6	99	99
	Reichenbachiella agariperforans	0.61	5	99	92
	Bradyrhizobium spp.	0.61	5	99	100
	Paenibacillus spp.	0.61	5	99	99
	Total		824		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A2 (t1, 19h)	*Komagataeibacter* spp.	97.45	1391	99	99
	Pelomonas puraquae	1.19	17	100	99
	Halomonas spp.	0.77	11	99	100
	Undibacterium oligocarboniphilum	0.63	9	99	100
	Total		1428		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A3 (t3, 23h)	*Komagataeibacter* spp.	83.9	6818	99	100
	Pantoea spp.	3.92	320	100	99
	Paenibacillus spp.	5.93	516	100	99
	Pseudomonas aeruginosa	3.20	261	100	100
	Acinetobacter spp.	3.10	251	95	99

	Total		8166		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A4 (t4, 29h)	*Komagataeibacter* spp.	100.0	26869	99	100
	Total		26869		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A5 (t5, 36h)	*Komagataeibacter* spp.	84.72	1524	100	99
	Malvídeas, cloroplasto	2.55	47	100	99
	Enterobacteriaceae	2.82	52	100	99
	Caulobacter spp.	1.41	26	100	99
	Desulfobulbus spp.	1.35	25	100	100
	Staphylococcus spp.	1.95	36	100	99
	Bradyrhizobium spp.	1.08	20	100	100
	Campylobacter gracilis	1.03	19	100	100
	Fusobacterium nucleatum	0.92	17	100	100
	Corynebacterium vitaeruminis	0.81	15	100	99
	Acinetobacter spp.	0.70	13	95	100
	Pseudomonas spp.	0.65	12	95	99
	Total		1806		

16S rRNA gene region 2:

Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A1 (t0, 0h)	*Komagataeibacter* spp.	89.93	3536	100	100
	Staphylococcus spp.	2.67	105	100	100
	Acinetobacter spp.	1.7	67	100	100
	Uncultured spirochete	1.4	55	100	97
	Burkholderiales	1.22	48	100	100
	Ralstonia spp.	0.79	31	100	100
	Oenococcus oeni	0.79	31	100	99
	Propionibacterium acnes	0.76	30	99	100
	Pseudomonas spp.	0.73	29	100	100
	Total		3932		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A2 (t1, 19h)	*Komagataeibacter* spp.	97.88	9079	100	100
	Oenococcus oeni	0.86	80	100	100
	Propionibacterium acnes	0.62	58	100	99
	Staphylococcus spp.	0.62	58	100	100
	Total		9275		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A3 (t3, 23h)	*Komagataeibacter* spp.	87.79	8770	100	100
	Paenibacillus spp.	7.32	732	100	100
	Enterobacteriaceae	2.45	245	100	100

	Pseudomonas spp.	1.68	168	98	100
	Bacillus spp.	0.75	75	100	100
	Total		9990		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A4 (t4, 29h)	*Komagataeibacter* spp.	100	19819	99	100
	Total		19819		
Sample	**Species**	**Results in**			
		(%)	**(nº seqs)**	**% coverage**	**% identity**
A5 (t5, 36h)	*Komagataeibacter* spp.	93.93	2800	100	100
	Paenibacillus spp.	1.81	54	100	100
	Fusobacterium nucleatum	1.64	49	100	99
	Enterobacteriaceae	0.97	29	100	99
	Propionibacterium acnes	0.72	26	100	99
	Staphylococcus spp.	0.77	23	100	100
	Total		2981		