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Taxonomy, characterization and application of the honey bee-associated genus *Bombella*

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List of Abbreviations

(v/v)	Volume per volume
(w/v)	Weight per volume
°C	Degree celcius
μ_{max}	Maximum growth rate
AAB	Acetic acid bacteria
ACN	Acetonitrile
AF4	Asymmetric flow field-flow fractionation
ALDH	Alcohol dehydrogenase
ANI	Average nucleotide identity
B.	*Bombella*
BADGE	Blast Diagnostic Gene Finder
bp	Base pairs
$CaCO_3$	Calcium carbonate
CFU	Culture forming units
COG	Clusters of Orthologous Genes
DH	Dehydrogenase
H_2O_d	Distilled water
E.	*Escherichia*
ED	Entner-Doudoroff
EMP	Embden–Meyerhof–Parnas
EPS	extracellular polysaccharides
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	Formic acid
FAD	Flavin adenine dinucleotide
fdr	False discovery rate
Fru*f*	Fructofuranose
FTase	Fructosyltransferase
g	Gram
× g	Times centrifugal force
G.	*Gluconobacter*

Ga.	*Gluconacetobacter*
GH	Glycoside hydrolase
h	Hour
HMW	High molecular weight
HPEAC	High-performance anion exchange chromatography
IJSEM	International Journal of Systematic and Evolutionary Microbiology
L	Liter
LB	Lysogeny broth
M	Molar
m/z	Mass over charge
MALLS	Multi-angle laser light scattering
MFS	Major facilitator superfamily
MWCO	Molecular weight cut off
MS	Mass spectrometry
ms	Millisecond
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
OD	Optical density
ORF	Open reading frame
P.	*Paenarthrobacter*
PAD	Pulsed amperometric detection
PCR	Polymerase chain reaction
PGAP	Prokaryotic Genome Annotation Pipeline
PMAA	Permethylated alditol acetate
ppm	Parts per million
PPP	pentose phosphate pathway
PQQ	pyrrolo-quinolinequinone
rRNA	Ribosomal ribonucleic acid
s	Seconds

sp.	Species
spp.	Species (plural)
T1PKS	Type 1 polyketide synthase
T4SS	Type IV secretion system
TCA	Tricarboxylic acid
TFA	Trifluoroacetic acid
UV	Ultraviolet

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1 Introduction

1.1 Acetic acid bacteria

Acetic acid bacteria (AAB) have a long history in microbiology research. The first strain of the genus *Acetobacter* was isolated and described by Martinus Willem Beijerinck from "Mother-Vinegar" back in the 19th century (Bourgeois and Barja 2009). To date, 47 genera have been validly described in the family *Acetobacteraceae*[1], to which the AAB belong along with the so-called acidophilic bacteria. In 2021, there were 44 genera and 177 valid species, divided into 19 genera and 97 species of AAB and 25 genera and 80 species of acidophilic bacteria (Parte et al. 2020; Guzman and Vilcinskas 2022).

AAB are Gram-negative or Gram-variable, catalase positive, oxidase negative, non-spore forming, motile or non-motile, ellipsoidal to rod-shaped cells that can occur single, in pairs or chains with sizes varying between 0.4-1 μm wide and 0.8-4.5 μm long. AAB have an obligate aerobic metabolism. The growth temperature range is 5-42°C with optima between 25-30°C. The optimum pH for the growth of AAB is 5.0-6.5, while they can grow at acidic pH values between 3.0-4.0 (Sievers and Swings 2005; Sengun 2016).

Several characteristics have been described for AAB that distinguish them from other bacteria. The best known ability is the formation of acetic acid from ethanol in a two-step conversion. In this process, the enzymes alcohol dehydrogenase (DH) and aldehyde DH are involved, which are membrane-bound enzymes with their catalytic sites in the periplasm. The enzymatic conversion therefore takes place outside the cells, whereby the majority of the product is secreted into the extracellular milieu (Illeghems et al. 2013; Azuma et al. 2009; Prust et al. 2005; Raspor and Goranovic 2008; Deppenmeier et al. 2002). Another characteristic of AAB, mainly attributed to the genus *Gluconobacter*, is the incomplete oxidation of sugars or sugar alcohols (Deppenmeier and Ehrenreich 2009). These oxidation reactions are also catalyzed by membrane-bound DHs. The membrane-bound DHs involved in these so-called oxidative fermentations are coenzyme-dependent and coupled to the respiratory chain by the transfer of electrons to ubiquinone. The reduced product ubiquinol in turn is an electron donor for ubiquinol oxidases of

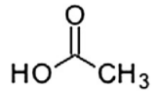
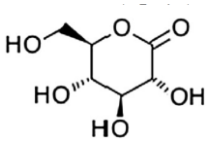
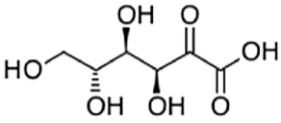
[1] https://lpsn.dsmz.de/family/acetobacteraceae (accessed: 18.11.2023)

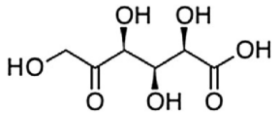
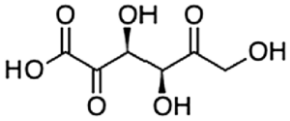
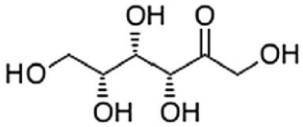
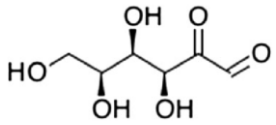
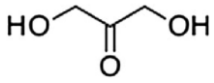
the respiratory chain, which are involved in energy production (Matsushita et al. 1994; Adachi et al. 2003; Roos and Vuyst 2018; Deppenmeier and Ehrenreich 2009).

1.2 Molecules with biotechnological applications produced by AAB

The oxidation potential of membrane-bound DHs from AAB are used in a variety of industrial applications. An example is the extracellular conversion from glucose to glucono-delta-lactone which is catalyzed by a glucose DH. Glucono-delta-lactone is stable under acidic conditions, but can spontaneously hydrolyze to gluconic acid under neutral and alkaline conditions or can be converted to gluconic acid by a membrane-bound gluconolactonase (Ameyama et al. 1981; Matsushita et al. 1994; La China et al. 2018). Another example of biotechnologically relevant molecules produced by AAB is the conversion of D-sorbitol to L-sorbose, which is necessary for the production of L-ascorbic acid (vitamin C) (Prust et al. 2005; Deppenmeier et al. 2002). Table 1 shows a selection of relevant molecules produced by AAB, as well as their precursor molecule, catalyzing enzyme and industrial areas of application.

Table 1: Selection of molecules with industrial applications produced by AAB, their precursor molecule, catalyzing enzyme and industrial applications (modified from La China et al. 2018).

Molecule	Precursor	Catalyzing enzyme	Areas of application
 Acetic acid	Acetaldehyde	Aldehyde DH	Food, chemical
 Glucono-delta-lactone	Glucose	Glucose DH EC 1.1.5.2	Food, chemical, pharmaceutical
 2-Keto-D-gluconate	Gluconic acid	Gluconate DH EC 1.1.99.3	Food, chemical, pharmaceutical

 5-Keto-D-gluconate	Gluconic acid	Glycerol DH EC 1.1.99.22	Food, chemical, pharmaceutical
 2,5-Diketo-D-gluconate	2-Keto-gluconate	Dehydrogluconate DH EC 1.1.99.4	Food, chemical, pharmaceutical
 L-sorbose	D-sorbitol	Glycerol DH EC 1.1.99.22	Pharmaceutical, cosmetic, food
 L-Sorbosone	L-Sorbose	Sorbose DH EC 1.1.99.12	Pharmaceutical, cosmetic, food
 Dihydroxyacetone	Glycerol and other polyols	Glycerol DH EC 1.1.99.22	Pharmaceutical, chemical, cosmetic

1.3 AAB in food fermentations

The unique ability of acetic acid bacteria to incompletely oxidize sugars and alcohols through membrane-bound DHs outside the cytoplasm has made them important organisms in the fermentation of foods. As mentioned above, best known is the conversion of ethanol into acetic acid, which is used in vinegar fermentation. The formation of acetic acid by AAB also plays an important role in cocoa fermentation. Complex interaction with lactic acid bacteria (LAB) and yeasts leads to a natural fermentation that makes cocoa beans usable, e.g. for chocolate production (Vuyst et al. 2010; Papalexandratou et al. 2011; Schwan 1998).

AAB also play an important role in the production of fermented beverages like kombucha, water kefir and lambic beer (Roos and Vuyst 2018). Kombucha, for example, is a drink with the basic ingredients water, sugar and tea. By adding the starter culture, a non-alcoholic drink with a sour

taste is fermented at room temperature within 1 to 3 weeks. The starter culture cells are contained in a so-called tea fungus, which is composed of cellulose and therefore floats, creating direct contact with oxygen. As in cocoa fermentation, kombucha fermentation is driven by the interaction of AAB, LAB and yeasts (Reva et al. 2015; Marsh et al. 2014). Besides the formation of aroma compounds, AAB such as *Komagataeibacter xylinus* are responsible for the formation of the cellulose network (Jayabalan et al. 2014; Tan et al. 2012).

An example of a more modern use of acetic acid bacteria in the food industry is "Bionade". Here, sugars obtained from barley malt are converted into gluconate by a *Gluconobacter oxydans* strain. This fermented solution serves as the basis for the production of the lemonade-like beverage and provides its distinctive taste (Kowalsky et al. 2011).

1.4 Adaptation of AAB to food fermentations and biotechnological processes

In food fermentations and biotechnological processes, random mutagenesis of starter culture strains is a method to achieve better results such as higher yields, fewer side products or more resistant strains (Wu et al. 2015; Liang et al. 2014; Mladenović et al. 2019). Over the years, methods other than UV irradiation or the use of chemical mutagens have been developed, such as atmospheric and room temperature plasma (ARTP)-intermediated mutagenesis, which is more user-friendly, more efficient and also safer (Zhang et al. 2014; Yu et al. 2020).

Several studies on mutagenesis-based adaptation of AAB strains have been published. An example for an improved biotechnological process was the adaption of a *G. oxydans* strain to increase the yield of 1,3-dihydroxyacetone (Lin et al. 2016). The authors used a combined mutagenesis, including UV irradiation, ARTP and ion beam implantation. The mutant strain achieved a 115.7 % higher productivity and the cultivation time could be reduced from 54 h to 36 h. In another study, an industrial *Acetobacter pasteurianus* strain was adapted to high ethanol concentrations to increase the yield of acetic acid, which is relevant for the production of vinegar (Wu et al. 2015). The mutant strains could persist in 11 % (v/v) ethanol and reached a 385.7 % higher acetic acid yield than the original strain.

1.5 Safety aspects of AAB

As described above, AAB play an important role in fermented foods and have done so for centuries. Therefore, most AAB genera can be considered as safe for consumption. On the list of "Microorganisms & Microbial-Derived Ingredients Used in Food", the U.S. Food and Drug Administration (FDA) lists several ingredients derived from AAB as generally regarded as safe (GRAS)[2]. An example is glucono-delta-lactone from *Acetobactor suboxydans* (§184.1318).
To date, there are rare cases where AAB have been opportunistic pathogens for people with pre-existing conditions or chronic diseases. An infection by *Asaia bogorensis* has been reported in 2004 in a patient with a peritoneal dialysis catheter (Snyder et al. 2004). Other infections resulting from AAB have been caused by *Granulibacter bethesdensis* strains in patients with chronic granulomatous disease (Greenberg et al. 2006). Nevertheless, food fermentations by AAB can be assumed to be safe.

1.6 Formation of extracellular polysaccharides by AAB

AAB can form various extracellular polysaccharides (EPS). One example already mentioned is cellulose, which gives the tea fungus its structure and is also relevant for the formation of mother of vinegar (Aykın et al. 2015; Kaushal and Walker 1947). Figure 1 shows photos of a cellulose-containing mother of vinegar taken from a commercial apple cider vinegar.

Figure 1: Mother of vinegar from a commercial apple cider vinegar[3].

[2] FDA (2018). Microorganisms & Microbial-Derived Ingredients Used in Food (Partial List). U.S. Food and Drug Administration. Accessed: 16.02.2024. https://www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list
[3] Pictures by Luca Härer, recorded on 02.03.2024.

Cellulose belongs to the homopolysaccharides and consists of individual *β*-(1→4)-linked glucan chains that are assembled into fibrils (Chawla et al. 2009). In *Acetobacter xylinum*, the cellulose synthesizing complex is well described. It is composed of several subunits that span the outer and inner cell membranes and uses intracellular UDP-glucose as a substrate (Endler et al. 2010; Hu et al. 2010). Although bacterial and plant cellulose have a similar chemical structure, bacterial cellulose has different properties, such as higher crystallinity and lower degree of polymerization (Bielecki et al. 2005). Because of this unique structure and insolubility in water, bacterial cellulose is suitable for many applications, including filter membranes, wound dressings, and emulsion stabilizers (Blanco Parte et al. 2020).

Other homopolysaccharides that are produced by AAB are levans. The fructofuranose (Fru*f*) backbones of levans consists of *β*-(2→6) linkages (Figure 2), which distinguishes levans from inulins which are composed of *β*-(2→1) Fru*f* linkages (Velázquez-Hernández et al. 2009).

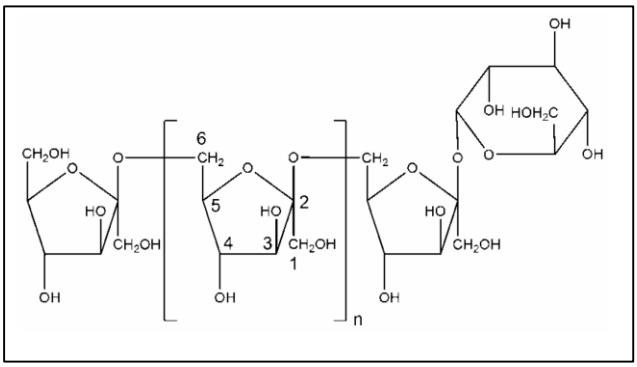


Figure 2: Schematic structure of levans from AAB (Jakob et al. 2013).

The enzymes that catalyze the formation of levans from sucrose are called levansucrases. They are classified as hydrolase GH 68 family enzymes by the "Carbohydrate Active Enzyme Database" (CAZy; http://www.cazy.org). While the synthesis of cellulose requires several protein units that are integrated into both membranes, levansucrases are present extracellularly and are monomers (Jakob et al. 2019; Velázquez-Hernández et al. 2009). Levansucrases are found in a variety of AAB, like *Zymomonas mobilis*, *Gluconacetobacter diazotrophicus, Gluconobacter albidus* and *Kozakia baliensis* (Jakob et al. 2019). Bacterial fructans offer many applications, for example as prebiotics in foods or infant formulas (Kaur and Gupta 2002; Coussement 1999; Srikanth et al. 2015).

1.7 Habitats of AAB

AAB are often isolated from environments in which sugars and/or alcohols are available. Such habitats can be flowers or pollen from which for example *Swingsia samuiensis, Gluconobacter thailandicus* or *Asaia prunellae* were isolated (Malimas et al. 2013; Tanasupawat et al. 2004; Suzuki et al. 2010). Another example of habitats from which acetic acid bacteria can be isolated are fruits such as apples (*Komagataeibacter swingsii),* strawberries (*Gluconobacter frateurii)* or peaches (*Acetobacter persici*) (Dellaglio et al. 2005; Mason and Claus 1989; Iino et al. 2012). Naturally, there are many species that have been isolated from food fermentations like *Acetobacter pomorum* from vinegar, *Gluconobacter cerevisiae* from lambic beer or *Acetobacter ghanensis* from a cocoa fermentation (Sokollek et al. 1998; Spitaels et al. 2014; Cleenwerck et al. 2007).

1.8 AAB and insects

Other sugar-rich habitats where AAB can be found are insects that rely on a sugar-based diet, particularly those in the orders *Diptera* (Flies), *Hymenoptera* (sawflies, wasps, bees, and ants) and *Hemiptera* (true bugs) (Crotti et al. 2010). Many insects have gastrointestinal tracts with an oxic environment and few anoxic niches, providing a favorable environment for aerobic bacteria that can cope with micro-oxic conditions (Cox and Gilmore 2007; Sudakaran et al. 2012). Studies on the gut microbiota associated with *Drosophila* or *Apis* spp. confirmed the absence of obligate anaerobic bacteria and the presence of aerobic, facultatively aerobic, or aerotolerant bacteria (Mohr and Tebbe 2006; Cox and Gilmore 2007).

Bacteria that colonize insect hosts can be transmitted vertically, i.e. from the parents to the offspring (Damiani et al. 2008). However, horizontal transmissions are also possible. It was shown that a strain of the genus *Asaia* isolated from mosquitoes can colonize a phylogenetically distant leafhopper species. The bacteria were administered to the leafhoppers with their food and the distribution in the body was examined using fluorescence (Crotti et al. 2009). Such transmission of AAB could occur, for example, on flowers where different bee species collect pollen or on fermented/rotten fruits that attract *Drosophila* species due to the smell of vinegar produced by AAB (Crotti et al. 2010; Mazzetto et al. 2016). These interactions between insects, microorganisms and plants can also be a problem in agriculture, as in the case of grape sour rot. It is assumed that AAB are transferred to the affected grapes by insects such as *Drosophila*. This can lead to interactions

with yeasts, similar to vinegar production, resulting in the formation of acetic acid and spoilage of the grapes (Hall et al. 2018).

Some symbiotic properties have been described for AAB in relation to the colonized insects. It is assumed, for example, that the extremely carbon-rich diet of insects should lead to a nitrogen deficiency, which can be compensated for by the uptake of nitrogen-fixing bacteria (Nardi et al. 2002). Several examples of nitrogen-fixing strains have been reported among AAB (Dutta and Gachhui 2006, 2007; Fuentes-Ramírez et al. 2001). In another study, the influence of an *Asaia* strain on the development of the larvae of *Anopheles stephensi* (mosquito species) was investigated. The larvae were treated with antibiotics and then the bacterial strain was supplemented. The larvae supplemented with the *Asaia* strains showed a similar development to the control group without treatment, while the development of the larvae without *Asaia* supplementation was impaired (Chouaia et al. 2012). It could thus be shown that AAB can have a considerable influence on the development of insects.

1.9 *Bombella* spp. are symbionts of the western honey bee *Apis mellifera*

Other AAB that are considered insect symbionts are strains of the genus *Bombella*. Various studies have described positive influences of the bacteria on the western honey bee *Apis mellifera*. Honey bee symbionts are of special interest because their hosts are essential for the pollination of crops, a billion-dollar industry that is struggling with the consequences of pollinator decline (Gallai et al. 2009).

As for *Asaia* spp. in mosquito larvae, a positive influence on the survival of the honey bee larvae was observed *in vitro* for *Bombella apis.* In the corresponding study, the effect of supplementing the larvae with *Bombella apis* and *Escherichia coli* on larvae mortality was tested. Two out of three *B. apis* strains had a significant positive effect on mortality, while the mortality of the larvae supplemented with *E. coli* was not changed compared to the reference (Corby-Harris et al. 2014).

In a different study, various *B. apis* strains were tested for their ability to suppress the growth of two insect fungal pathogens, *Beauveria bassiana* and *Aspergillus flavus*. All strains reduced the growth of the fungal pathogens *in vitro.* In addition, *in vivo* experiments showed that bee broods supplemented with *B. apis* were less vulnerable to *A. flavus* infections. The authors assume that the *B. apis* strains secrete an antifungal metabolite, since filtered culture supernatant without cells had a negative influence on the growth of the fungal pathogens (Miller et al. 2021).

Besides the positive influence on larvae development and fungal pathogen defense, symbiotic traits have also been described for *B. apis* in relation to honey bee nutrition. The authors showed that *Bombella* strains are the only tested bacteria that can grow in the antimicrobial environment of the honey bee larval diet. It was also shown that *B. apis* can produce all essential amino acids and thus influence the composition of the larval diet. In particular, the concentration of the amino acid lysine is significantly increased. In experiments with developing honey bee larvae, it was shown that supplementation with *B. apis* strains protects the larvae from mass reduction due to nutrition limitations (Parish et al. 2022). Besides the influence on the larvae's nutrition, amino acids from *Bombella* strains seem to have an influence on the queen bee. Li et. al (2023) showed that *Bombella* colonizing the queen bee's gut microbiota may regulate the queen's ovarian metabolism through tryptophan metabolism.

Bombella spp. strains were found in several niches in the *A. mellifera* beehive, such as crop and hindgut of the worker bees, the gut of the queen, larvae and royal jelly (Tarpy et al. 2015; Anderson et al. 2013; Corby-Harris et al. 2014; Smutin et al. 2022). In further microbiome studies, *Bombella* strains were also found in other bees, such as *Bombus terrestris, Bombus lapidarius, Apis dorsata, Caupolicana yarrow* or *Xylocopa californica* (Martinson et al. 2011; Tang et al. 2022). Unfortunately, no studies are known that deal with the symbiosis of *Bombella* spp. with bees other than the western honey bee *A. mellifera*. Therefore, no statement can be made as to whether *Bombella* spp. are found in other bee colonies through horizontal transmission, e.g. via pollinated flowers, or whether they have symbiotic functions there.

1.10 *Bombella* spp. taxonomy

The valid description of the genus *Bombella* was based on the strain *B. intestini* R52487^T isolated from the gut of the red-tailed bumble bee *B. lapidarius* in Belgium (Li et al. 2015). *B. apis* was described as the second valid species, the corresponding type strain *B. apis* MRM1^T was isolated from the midgut of a western honey bee *A. mellifera* in South Korea (Yun et al. 2017). In another publication, the species *Bombella mellum* and *Bombella favorum* were validly described. Both type strains *B. mellum* TMW 2.1889^T and *B. favorum* TMW 2.1880^T were isolated from the environment of the western honey bee *A. mellifera* in Freising, Germany (Hilgarth et al. 2021).

In earlier studies involving strains of the *Bombella* genus, the name *Parasaccharibacter* was used. In fact, the description of the genus *Parasaccharibacter* was published before the genus *Bombella*,

but not in accordance with the International Journal of Systematic and Evolutionary Microbiology (IJSEM) standards and therefore not valid (Corby-Harris et al. 2014). Smith et al. (2021) have analyzed the phylogeny of several published genomes and were able to assign all *Parasaccharibacter* strains to the species *B. apis*. In addition, several *Saccharibacter* strains that had been incorrectly assigned due to similar 16S rDNA could be assigned to *B. apis*. Unfortunately, the name *Parasaccharibacter* can still be found in new publications, although it should no longer be used (Alberoni et al. 2023; Santorelli et al. 2023).

Another name used in some publications instead of *Bombella* spp. is Alpha 2.2. This name was established primarily through microbiome studies in which respective strains could be assigned to a clade of the *Alphaproteobacteriae* (clade Alpha-2) on the basis of their 16S rRNA genes, but not yet to any genus or species (Babendreier et al. 2007; Anderson et al. 2013).

1.11 Aims of this work

Although initial attempts have been made to clarify the taxonomy and phylogeny of the genus *Bombella*, there are still other genomes with unclear taxonomy in the NCBI database. Therefore, in the first part of this work, the corresponding genomes were analyzed phylogenetically in order to obtain a clear picture of the genus-wide phylogeny.

The genome data was then used for an interspecies comparative genomics analysis in which typical properties of acetic acid bacteria, such as membrane-bound dehydrogenases, were investigated. In addition, characteristics that can be attributed to an adaptation to the sugar-rich beehive habitat were analyzed.

A known characteristic of *Bombella* strains, which probably represents an evolutionary adaptation to the habitat, is the high tolerance to glucose. Most type strains can grow in medium containing 500 g/L glucose, while concentrations of up to 400 g/L glucose can be found in honeycombs (Hilgarth et al. 2021; Ball 2007). Other AAB are considered osmotolerant if they can tolerate glucose concentrations of up to 300 g/L (Yukphan et al. 2008; Yukphan et al. 2005). In this work, all available *Bombella* strains from our strain collection were tested for their tolerance to high glucose concentrations in order to identify possible differences within the species.

To investigate the cellular mechanisms leading to high glucose tolerance in *Bombella* spp., a combination of a proteomics and a metabolomics experiment was done with *B. favorum* TMW 2.1880 as a representative strain. In addition, it was investigated whether the cellular response to

NaCl stress is different than to glucose. This was done because both molecules lower the A_w-value of the medium but in contrast to glucose, *Bombella* strains are sensitive to NaCl (max. 15 g/L NaCl) (Hilgarth et al. 2021).

After examining fundamental research topics such as taxonomy, habitat adaptation and tolerance mechanisms in the first part of this work, the focus of the second part was on potential industrial applications of *Bombella* strains. The properties of AAB, such as the extracellular formation of organic acids from sugars, suggest that they are suitable for the fermentation of fruit juices to produce non-alcoholic, acidic and refreshing beverages as alternatives to soft drinks (Roos and Vuyst 2018). Strains of *Bombella* spp. could be particularly suitable, as they have a high sugar tolerance and the symbiosis with honey bees creates positive associations with potential consumers. In addition, such an innovative beverage could provide support for the german fruit juice industry, which is struggling with the decline in consumption of "classic" fruit juices (Figure 3).

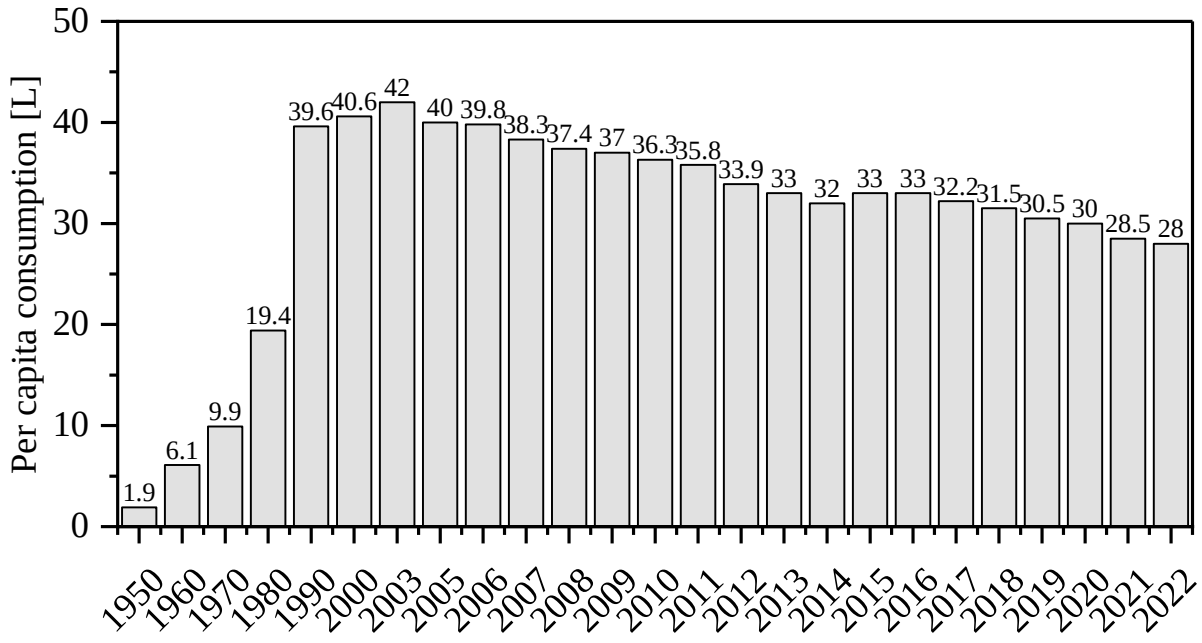


Figure 3: Per capita consumption of fruit juice and nectar in Germany by 2022[4].

To lay the groundwork for possible beverage fermentations, the growth of available *Bombella* strains was tested in various fruit juices. It was also tested whether a *Bombella* strain can be adapted

[4] wafg. (2023). Pro-Kopf-Konsum von Fruchtsaft und -nektar in Deutschland in den Jahren 1950 bis 2022 (in Liter). Statista. Statista GmbH. Accessed: 23.11.2023. https://de.statista.com/statistik/daten/studie/76851/umfrage/pro-kopf-verbrauch-von-fruchtsaft-in-deutschland-seit-2000/

to the fruit juice milieu by UV mutagenesis. In addition, a proteomics study was performed to determine which cellular functions were affected by UV mutagenesis.

Although many AAB form EPS with industrial applications such as cellulose or levan, there are no descriptions of *Bombella* spp. associated with EPS formation yet. Therefore, in the last part of the work, it was investigated whether available Bombella strains form EPS when grown on sucrose-containing agar plates.

2 Materials and Methods

2.1 Microbiological methods

2.1.1 Bacterial strains and cultivation conditions

All strains were generally cultivated at oxic conditions. If not stated otherwise, AAB (*Gluconobacter* and *Bombella* strains) were cultivated at 30 °C in LMG404 media (50 g/L glucose, 10 g/L yeast extract (YE), pH 6.6), *E. coli* Top10 at 37 °C in lysogeny broth (LB) media (10 g/L tryptone, 5 g/L YE and 5 g/L NaCL, pH 7). If necessary, 100 µg mL^{-1} ampicillin was added to the media. For some experiments *Bombella* spp. were cultivated in salt stress medium (50 g/L glucose, 10 g/L YE and 10 g/L NaCL, pH 6.6) or in glucose stress medium (400 g/L glucose, 10 g/L yeast extract (YE), pH 6.6).

In general, shaking flasks were used for cultivation at 200 rpm. For bacterial cultures with volumes less than 300 µL, f-bottom 96-well plates were used, which were shaken at 500 rpm. All bacterial strains used in this work are summarized in Table 2.

Table 2: Bacterial strains used in this work. TMW: designation in the chair's own strain collection.

Strain designation	TMW	Reference	Standard medium
Bombella apis MRM1^T	2.2221	(Yun et al. 2017)	LMG 404
B. apis TMW 2.1882	2.1882	this work	LMG 404
B. apis TMW 2.1884	2.1884	this work	LMG 404
B. apis TMW 2.1886	2.1886	this work	LMG 404
B. apis TMW 2.1888	2.1888	this work	LMG 404
B. apis TMW 2.1890	2.1890	this work	LMG 404
B. apis TMW 2.1891	2.1891	this work	LMG 404
Bombella intestini R52487^T	2.2220	(Li et al. 2016)	LMG 404
Bombella favorum TMW 2.1880^T	2.1880	(Hilgarth et al. 2021)	LMG 404
Bombella mellum TMW 2.1889^T	2.1889	(Hilgarth et al. 2021)	LMG 404
Bombella pluederhausensis TMW 2.2543	2.2543	this work	LMG 404
Bombella pollinis TMW 2.2556	2.2556	this work	LMG 404
Bombella saccharophila TMW 2.2558	2.2558	this work	LMG 404

Bombella dulcis TMW 2.2559	2.2559	this work	LMG 404
B. favorum TMW 2.1880 (adapted to 400 g/L Glucose)	2.2724	this work	LMG 404
Gluconobacter oxydans DSM46615	2.874	-	LMG 404
Escherichia coli TOP10	2.580	-	LB

2.1.2 Bacterial growth monitoring

Growth was either monitored via the optical density (OD) or culture forming units per milliliter (CFU/mL). The OD was measured in cuvettes or in case of 96-well plates directly in a plate photometer (SPECTROstar^{nano}, BMG Labtech, Ortenberg, Germany). To determine the CFU/mL, suitable dilutions were spread in triplicate on agar plates. The colonies were counted manually.
For the analysis of the maximal growth rates μ_{max} the R package "grofit" was used (Kahm et al. 2010) in R studio.

2.1.3 16S rRNA gene sequencing

Taxonomic relationships between strains were analyzed by partial amplification of the 16S rRNA gene sequence. For that purpose, the genomic DNA of the respective strain was isolated and purified using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek, Norcross, USA) according to the manufacturer's instructions. Next, the 16S rRNA gene was partially amplified using the PCR kit "Taq Core Kits 10" (MP Biomedicals Inc., Eschwege, Germany) and the standard primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1507R (5' TAC CTT GTT ACG ACT TCA CCC CAG 3') according to the manufacturer's instructions. The amplified DNA was purified using the Monarch PCR DNA Cleanup Kit (New England Biolabs GmbH (NEB), Frankfurt a.M., Germany) and externally sequenced (Eurofins Genomics, Ebersberg, Germany).

2.1.4 Physiological characterization

Strains were grown on LMG 404 agar plates at temperatures between 10 °C and 45 °C in 5 °C intervals to determine temperature tolerance and optimum. Growth was observed after 24 h, 48 h and 72 h. Growth at different pH values and glucose concentrations was tested in 96-well plates over 72 h in a plate photometer (SPECTROstar^{nano}, BMG Labtech, Ortenberg, Germany). The pH range tested was between 3.5 and 10 in 1 pH intervals in buffered LMG 404 media. Citrate buffer was used for pH 3.5 – 6, phosphate buffer for pH 7 – 8, tris buffer for pH 9 and carbonate buffer

for pH 10. For glucose experiments, growth was monitored in media containing 1 % (w/v) yeast extract with 1, 5, 10, 20, 30, 40, 45 and 50 % (w/v) glucose. The halo tolerance of *Bombella* spp. was tested on LMG 404 agar plates with NaCl concentrations ranging from 0.5 % to 3 % (w/v) in 0.5 % intervals at 30 °C for 72 h.

The formation of pellicles was tested in static LMG 404 cultures at 30 °C for 7 days. Assimilation of ammonium nitrogen was tested on Hoyer's medium modified by Frateur (Shimwell 1957) with 1 % (w/v) glucose and ethanol 3 % (v/v) as carbon sources. Oxidation of glycerol to dihydroxyacetone was tested according to (Aydin and Aksoy 2009). Production of acetate from ethanol was tested on agar plates containing $CaCO_3$ (Shimwell et al. 1960).

2.1.5 Analysis of antibiotic resistance

The resistance of strains against antibiotics was tested with a disk diffusion method, adapted from the "European Committee on Antimicrobial Susceptibility Testing" (EUCAST) guidelines. Overnight cultures were spread on LMG 404 media agar plates and antibiotic discs (Oxoid Ltd, Cheshire, UK) were placed onto the plates. The diameter of the growth inhibition zone was measured after 48 h of incubation at 30 °C at oxic conditions.

2.1.6 Strain adaption via UV mutagenesis

B. favorum TMW 2.1880 was used for mutagenesis experiments. The aim was to adapt the strain to high glucose concentrations to reach better growth in fruit juice concentrates. For the adaption, the strain was cultivated for 24 h in 10 mL of medium containing 400 g/L glucose. For the mutagenesis, the shaking flask containing the culture was placed on a UV light for 10 minutes. Then, 500 µL of the treated culture were added to fresh medium containing 400 g/L glucose. These steps were repeated 50 times. The so treated culture was then spread on a LMG 404 agar plate. After incubation for 2 days at 30 °C a single colony was used to inoculate 10 mL fresh LMG 404 media, resulting in the adapted strain.

2.2 Molecular biological methods

2.2.1 Expression plasmids

All plasmids used for heterologous gene expression are summarized in Table 3.

Table 3: Plasmids used for heterologous gene expression. All plasmids are based on a pBAD/*Myc*-His A plasmids with an Ampicillin resistance gene. TMW: designation in the chair's own strain collection.

Plasmid	Insert	Cloned from	Expression Host	TMW
pBAD-84	DTI93_RS00530	*B. apis* TMW 2.1884	*E. coli* TOP10	2.2726
pBAD-84ext	DTI93_RS00530 extended	*B. apis* TMW 2.1884	*E. coli* TOP10	2.2727
pBAD-89	CPA56_RS00775	*B. mellum* TMW 2.1889	*E. coli* TOP10	2.2728

2.2.2 Construction of expression plasmids

For heterologous expression of the *B. apis* TMW 2.1884 ORF DTI93_RS00530 and *B. mellum* TMW 2.1889 ORF CPA56_RS00775 in *E. coli* TOP10 the pBAD/*Myc*-His A plasmid was used. Additionally, an extended version (gene sequences in Supplementary Data S8) of the *B. apis* TMW 2.1884 ORF DTI93_RS00530 was cloned. *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889 genomic DNA was isolated using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek, Norcross, USA). To amplify the ORF DTI93_RS00530 the primer pair
(5‘-TTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGACGGACCTGTCCAATG-3‘) and
(5‘-GCTGGAGACCGTTTAAACTCAATGATGATGATGATGATGGCCATAATCACTTGTCGAAACGG-3‘)
was used. For the extended version the primer pair
(5‘-TTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGGTCACGATTCTTATTTTTG-3‘) and
(5‘-GCTGGAGACCGTTTAAACTCAATGATGATGATGATGATGGCCATAATCACTTGTCGAAACGG-3‘)
was used. Accordingly, the primer pair
(5‘-TTTCTCCATACCCGTTTTTTGGGCTAACAGGAGGAATTAACCATGATGGACCTGTCCAG-3‘) and

(5‘-GCTGGAGACCGTTTAAACTCAATGATGATGATGATGATGGCCGTAATCACTTGTA GAAACGG-3‘)

was used for the ORF CPA56_RS00775. A Q5 High-Fidelity DNA Polymerase (NEB, Frankfurt a.M., Germany) was used for the PCR according to the manufacturer’s instructions. The obtained PCR products were purified using a Monarch PCR and DNA cleanup kit (NEB, Frankfurt a.M., Germany). The unmodified pBAD/*Myc*-His A plasmid was digested using SalI-HF and NocI-HF restriction enzymes (NEB) according to the manufacturer’s instructions. In the same step, rSAP (NEB, Frankfurt a.M., Germany) was added for dephosphorylation of the of 5´-ends of the DNA. The linearized plasmids were separated from the inserts via agarose gel electrophoresis (1.4 % (w/v) agarose dissolved in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0)) and purified using the monarch DNA Gel Extraction Kit (NEB, Frankfurt a.M., Germany). For the integration of the amplified genes into the linearized plasmids, Gibson assembly (Gibson Assembly Master Mix, NEB) was performed according to the manufacturer’s protocol. The assembled expression plasmids were transformed into *E. coli* Top10 using heat-shock transformation (Froger and Hall 2007).

2.3 Genome and sequence analysis

2.3.1 Genomes

All bacterial genome data used in this work are summarized in Table 4.

Table 4: Genome data used in this work. BioSample identifiers are from the NCBI database.

***Bombella* strain**	**Reference**	**BioSample**
B. apis MRM1^{T}	(Yun et al. 2017)	SAMN16262074
B. apis ESL0368	(Bonilla-Rosso et al. 2019)	SAMN13280441
B. apis ESL0380	(Bonilla-Rosso et al. 2019)	SAMN13280444
B. apis ESL0387	(Bonilla-Rosso et al. 2019)	SAMN13280447
B. apis SME1	-	SAMN13042715
B. apis TMW 2.1882	this work	SAMN07674798
B. apis TMW 2.1884	this work	SAMN09635582
B. apis TMW 2.1886	this work	SAMN09635591
B. apis TMW 2.1888	this work	SAMN09641705
B. apis TMW 2.1890	this work	SAMN07675059
B. apis TMW 2.1891	this work	SAMN09635595

B. apis A29	(Corby-Harris et al. 2014)	SAMN04240487
B. apis AM169	(Chouaia et al. 2014)	SAMEA3139036
B. apis G773c	(Corby-Harris and Anderson 2018)	SAMN06649799
B. apis 3A1	(Veress et al. 2017)	SAMN05935507
B. apis M18	(Veress et al. 2017)	SAMN05935506
B. intestini R52487^{T}	(Li et al. 2016)	SAMN02598725
B. favorum TMW 2.1880^{T}	(Hilgarth et al. 2021)	SAMN07674723
B. mellum TMW 2.1889^{T}	(Hilgarth et al. 2021)	SAMN07674951
B. pluederhausensis TMW 2.2543	this work	SAMN29843715
B. pollinis TMW 2.2556	this work	SAMN29843716
B. pollinis. ESL0378	(Bonilla-Rosso et al. 2019)	SAMN13280442
B. pollinis ESL0385	(Bonilla-Rosso et al. 2019)	SAMN13280446
B. saccharophila TMW 2.2558	this work	SAMN29843717
B. saccharophila AS1	-	SAMN05720096
B. dulcis TMW 2.2559	this work	SAMN29843718

2.3.2 Genome sequencing

Genomic DNA was isolated from over-night cultures using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the instructions of the manufacturer. Genome sequencing (Eurofins Genomics, Constance, Germany) was done using an Illumina MiSeq platform. Whole genome assemblies were obtained using SPAdes (v. 3.90) (Huptas et al. 2016). The NCBI PGAP pipeline was used for genome annotations (Tatusova et al. 2016).

2.3.3 Comparative genomic tools

The BlAst Diagnostic Gene FindEr (BADGE) tool was used with default settings and a sequence identity cut-off of 70 % in order to identify similar genes in the different genomes (Behr et al. 2016). Annotations of interest were manually checked with RAST annotations (Aziz et al. 2008) or the InterPro web tool (Blum et al. 2021). Secondary metabolite biosynthesis gene clusters were identified with the antiSMASH webtool (Blin et al. 2021). For the annotation of carbohydrate active enzymes from the genome data the DBcan webserver (Yin et al. 2012; Zhang et al. 2018) was used. Sequence alignments were done with the Clustal Omega webtool (EMBL-EBI, Hinxton, UK) or the CLC Main Workbench (QIAGEN, Hilden, Germany).

2.3.4 Phylogenetic analysis

For phylogenetic analyses of genome data, the EDGAR 3.0 platform (Blom et al. 2009; Dieckmann et al. 2021) was used to build and align complete core genomes. Other sequence data, like peptide sequences, were aligned with the ClustalW tool integrated in MEGA X (Kumar et al. 2018). The alignments were then used to construct phylogenetic trees using the Neighbor-Joining method (Saitou and Nei 1987) in MEGAX.

Average nucleotide identities between all *Bombella* species were calculated using the orthoANI algorithm (Lee et al. 2016) implemented in OAT (Orthologous Average Nucleotide Identity Tool, version 0.93.1). The species delineation cut-off for orthoANI values is 95 %. *In silico* DNA-DNA hybridisation (isDDH) values were calculated via Formula 2 of the Type StrainGenome Server using the Genome-to-Genome Distance Calculator's subspecies concept (Meier-Kolthoff and Göker 2019; Meier-Kolthoff et al. 2013). Species delineation cut-off for isDDH is 70 % (Moore et al. 1987; Stackebrandt and Goebel 1994).

2.3.5 Protein sequence analysis

For the prediction of protein domains the InterPro webserver (Paysan-Lafosse et al. 2023) was used. Crystal structures from protein sequences were predicted with the SWISS-MODEL webserver (Waterhouse et al. 2018; Bienert et al. 2017; Guex et al. 2009; Studer et al. 2020; Bertoni et al. 2017).

2.3.6 Prediction of cellular protein functions

The eggNOG-mapper (Cantalapiedra et al. 2021; Huerta-Cepas et al. 2019) was used to predict the cellular functions of proteins based on sequence orthology. The proteins are grouped in so-called cluster of orthologous groups (COGs) (Tatusov et al. 1997). All possible COGs are summarized in Table 5.

Table 5: Cellular functions of all COGs and their abbreviations.

Abbreviation	COG
A	RNA processing and modification
B	Chromatin Structure and dynamics
C	Energy production and conversion
D	Cell cycle control and mitosis
E	Amino Acid metabolism and transport
F	Nucleotide metabolism and transport
G	Carbohydrate metabolism and transport
H	Coenzyme metabolism
I	Lipid metabolism
J	Translation
K	Transcription
L	Replication and repair
M	Cell wall/membrane/envelope biogenesis
N	Cell motility
O	Post-translational modification, protein turnover, chaperone functions
P	Inorganic ion transport and metabolism
Q	Secondary Structure
T	Signal Transduction
U	Intracellular trafficing and secretion
Y	Nuclear structure
Z	Cytoskeleton
R	General Functional Prediction only
S	Function Unknown

2.4 MALDI-TOF mass spectrometry

A MALDI-TOF mass spectrometry (MS) biotyper (Bruker Daltonics, Billerica, MA, USA) was used for the taxonomic identification of bacterial strains. Colonies were picked from agar plates with a sterile toothpick and spread on a MS target. The microorganisms were coated with a drop of 70 % (w/v) formic acid (FA). After all liquid was evaporated, the spots were covered with matrix solution. The matrix

solution was prepared by mixing HPLC grade $H2O_{dd}$, acetonitrile (ACN) and trifluoroacetic acid (TFA) in the ratio 19:20:1 and dissolving 10 mg/ml α-cyano-4-hydroxycinnamic acid. After the matrix solution was dried, the samples on the MS target were analyzed in the MALDI-TOF-MS biotyper.

2.5 Extracellular polysaccharide analysis

2.5.1 Production and recovery of fructans

For the synthesis of fructans, *Bombella* strains were plated on agar plates (pH 5) containing 10 g L^{-1} yeast extract, 50 g L^{-1} sucrose and 15 g L^{-1} agar-agar. For this purpose, 10 µL of an over-night culture was distributed equally on the agar plates. The plates were incubated at 30 °C for 4 days. Slime around the cultures indicated the formation of an extracellular polysaccharide. For heterologous fructan production, transformed *E. coli* Top10 was plated on modified LB-media agar plates containing additional 2 g L^{-1} arabinose for induction of the gene expression, 36 g L^{-1} sorbitol to prevent the formation of inclusion bodies and 50 g L^{-1} sucrose as a substrate. The plates were incubated at 20 °C for 2 weeks.

For the recovery of formed fructans, the bacteria and slime were washed off the plate with 1.5 mL saline (0.9 % NaCl) and the solution was collected. To obtain a higher yield, 10 plates of the same strain were used and the liquids were pooled. To remove the cells, the solution was centrifuged (10,000 × g, 10 min, 20 °C). Precipitation of the polysaccharides from the supernatant and dialysis were carried out according to Hundschell et al. (2020). The sample was treated with two volumes of chilled ethanol (-20 °C) to precipitate the polysaccharide. After centrifugation (10,000 × g, 10 min, 4 °C), the supernatant was discarded, and the pellet dissolved in H_2O_d. Dialysis against H_2O_d (MWCO: 3.5 kDa; 4 °C) was performed for 48 hours with at least five water changes. The dialyzed solution was then lyophilized to obtain the purified polysaccharide.

2.5.2 Monosaccharide composition analysis

Note: This method was performed by Luise Ernst at the Institute of Chemistry, Division of Food Chemistry of the Martin Luther University Halle-Wittenberg (Group of Prof. Daniel Wefers).

The monosaccharide composition of the fructans was analyzed after TFA hydrolysis. A first batch of the samples was hydrolyzed with 1 M TFA at 70 °C for 30 min for fructan hydrolysis. A second batch was hydrolyzed with 2 M TFA at 121 °C for 60 min to hydrolyze potentially present glucans, mannans or galactans. In both cases TFA was removed by evaporation and subsequent co-

evaporation with ethanol. Monosaccharides were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPEAC-PAD) on an ICS-6000 system (Thermo Scientific Dionex, Sunnyvale, CA, USA). Separation was achieved on a CarboPac PA20 column (150 mm×3 mm i.d., 6.5 µm particle size, Thermo Scientific Dionex) using a flow rate of 0.4 mL min^{-1}. Column equilibration was achieved with 10 mM NaOH for 15 min. After sample injection, the following gradient was applied: 0-20 min: 10 mM NaOH, 20-30 min: Linear gradient from 10 mM to 200 mM NaOH, 30-40 min: Isocratic 200 mM NaOH + 200 mM sodium acetate, 40-50 min: Isocratic 200 mM NaOH. The column temperature was 30 °C and the detector temperature was 25 °C. Quantification was achieved by using an external calibration.

2.5.3 Nuclear magnetic resonance spectroscopy

Note: This method was performed by Luise Ernst at the Institute of Chemistry, Division of Food Chemistry of the Martin Luther University Halle-Wittenberg (Group of Prof. Daniel Wefers).

For nuclear magnetic resonance (NMR) spectroscopy a 10 mg mL^{-1} fructan solution in D_2O was prepared and acetone was used as internal reference (^{1}H: 2.22 ppm, ^{13}C: 30.89 ppm according to Gottlieb et al. (1997). Proton, H,H-correlated spectroscopy, and heteronuclear single quantum coherence experiments were acquired on a 500 MHz DD2 spectrometer (Agilent, Santa Clara, CA, USA).

2.5.4 Methylation analysis

Note: This method was performed by Luise Ernst at the Institute of Chemistry, Division of Food Chemistry of the Martin Luther University Halle-Wittenberg (Group of Prof. Daniel Wefers).

Methylation analysis was conducted as described by Ernst et al. (2023). In brief, samples were solubilized in dimethyl sulfoxide, deprotonated with dry NaOH(s), and methylated by using methyl iodide. Permethylated levan was hydrolyzed with 1 M TFA at 70°C for 30 min, reduced by $NaBD_4$, and acetylated by using acetic anhydride and 1-methylimidazole. The obtained permethylated alditol acetates (PMAAs) were extracted into dichloromethane and separated by gas chromatography (GC). Identification was performed by using mass spectrometry and relative quantification was performed using a flame ionization detector and the molar response factors described by Sweet et al. (1975). Because the four PMAAs derived from 1,2- and 2,6-Fru*f* units have an identical retention time (in the case of the mannitol derivatives) or partially coelute (in the case of the sorbitol derivatives), their relative amounts were calculated based on the mass spectra.

For 1,2-Fru*f* units the intensities of specific fragments with m/z 161 and 190 were used to assess their portion. In the case of 2,6-Fru*f* units the intensities of fragments with m/z 162 and 189 were used for calculation.

2.5.5 Molecular weight determination via asymmetric flow field-flow fractionation

The molecular weight of purified fructans was determined using asymmetric flow field-flow fractionation (AF4, Wyatt Technology, Dernbach, Germany) coupled with multi-angle laser light scattering (MALLS, Dawn Heleos II, Wyatt Technology) and ultraviolet (UV) detection (Dionex Ultimate 3000, Thermo Fisher Scientific, Darmstadt, Germany) according to Ua-Arak et al. (2016, 2017). Separation was carried out on 10 kDa regenerated cellulose membranes (Wyatt Technology) using a 50 mM $NaNO_3$ eluent solution. Purified fructans were dissolved in H_2O_d (final concentration 0.1 g/L^{-1}) and injected (100 µL) into the separation channel. Each sample was measured at least twice. To calculate molar masses using UV concentration signals, the specific extinction coefficients of the isolated fructans at 400 nm were determined: A concentration series (0.1-10 mg mL^{-1} in dH_2O) of the purified fructans was prepared, and the UV extinctions at 400 nm were measured using a FLOUstar Omega microplate reader (BMG Labtech, Ortenberg, Germany). The obtained values were used to calculate the specific extinction coefficients [mL $(mg \cdot cm)^{-1}$] of the isolated levan samples. These coefficients were then used to calculate the molar mass distributions using the random coil model integrated in the ASTRA 6.1 software (Wyatt Technology, Goleta, CA, USA), assuming a refractive index increment (dn/dc) value of 0.146 mL g^{-1} (in 50 mM $NaNO_3$) for levan (Ua-Arak et al. 2017).

2.6 Proteomics

Proteomics experiments were conducted to analyze and compare the expression levels of intracellular proteins in different conditions. The experiments were conducted in cooperation with Susanne Wudy and Dr. Christina Ludwig from the Bavarian Biomolecular Mass Spectrometry Center (BayBioMS, Freising, Germany) of the Technical University of Munich.

2.6.1 Sample preparation

In order to prepare protein samples for analysis by mass spectrometry, the first step is to use an appropriate amount of cell culture. In preliminary experiments, a rule of thumb was developed for *Bombella* strains: 10 mL of culture with an OD of 1 gives approximately the desired protein

concentration of 1 g/mL in the final solution. This made it possible to calculate the volume of sample that was required during the experiments. The collected sample was centrifuged (8,000 × g, 10 min, 4 °C), washed with cold saline (0.9 % NaCl) and centrifuged again.

Cell lysis was performed according to Doellinger et al. (2020) and Abele et al. (2023). 100 µL of 100 % TFA was added to the pellet and the sample was incubated at 55 °C for 5 min. After that 900 µL neutralization buffer (2 M Tris) was added and the sample was vortexed. Neutral pH was checked with pH paper.

To check the protein concentration of the sample, the Pierce Coomassie (Bradford) Protein-Assay-Kit (Thermo Fisher Scientific, Darmstadt, Germany) was used. A calibration curve was calculated from a BSA standard with protein concentrations from 0.05 to 2 g/L. The determined protein concentration of the sample was used to calculate the volume for a total protein amount of 50 mg per sample. This volume was pipetted in a separate tube and stored at -80 °C until further analysis.

2.6.2 Trypsin digestion of protein samples

Note: These steps were performed at the BayBioMS.

The sample was then reduced (5 min at 95 °C) and alkylated (9 mM Tris(2-carboxyethyl)phosphine hydrochloride, 40 mM Chloroacetamide). Water-diluted samples (1:1; final concentration of 1 M Tris and 5 % TFA) were subjected to proteolysis with trypsin (0.4 µg trypsin for 20 µg protein input, 37 °C, overnight, 400 rpm). The enzymatic digestion was stopped by the addition of 3 % (w/v) FA.

Peptide samples were desalted using self-packed desalting tips prepared in-house using five discs of Empore C18 (3M) material stacked in 200 µL pipette tips (Rappsilber et al. 2007). The desalting tips were conditioned with 100 % ACN and 40 % ACN/0.1 % FA, and equilibrated with 2 % ACN, 0.1 % FA in HPLC grade water. Peptides were loaded, washed (2 % ACN, 0.1 % FA in HPLC grade water), and eluted (40 % ACN, 0.1 % FA in HPLC grade water). Samples were dried to completeness and re-dissolved in 13 µl 0.1 % FA in HPLC-grade water. Around 19 µg peptides (as determined by the Bradford assay) was injected into the mass spectrometer per run.

2.6.3 Data acquisition via Exploris – microflow label-free DDA

Note: These steps were performed at the BayBioMS.

Peptides were analyzed on a Vanquish Neo (microflow configuration) coupled to an Orbitrap Exploris 480 mass spectrometer (both Thermo Fisher Scientific, Darmstadt, Germany). Peptides

were applied onto a commercially available Acclaim PepMap 100 C18 column (2 µm particle size, 1 mm ID × 150 mm, 100 Å pore size; Thermo Fisher Scientific, Darmstadt, Germany) and separated using a stepped gradient with acetonitrile concentration ranging from 3 % to 24 % to 31 % solvent B (0.1 % FA, 3 % DMSO in ACN) in solvent A (0.1 % FA, 3 % DMSO in HPLC grade water) over 60 min. A flow rate of 50 µL/min was applied. The mass spectrometer was operated in data-dependent acquisition and positive ionization mode. MS1 full scans (360–1300 m/z) were acquired with a resolution of 60,000, a normalized automatic gain control target value of 100 %, and a maximum injection time of 50 ms. Peptide precursor selection for fragmentation was carried out at a 1.2 seconds cycle time. Only precursors with charge states from two to six were selected, and dynamic exclusion of 35 s was enabled. Peptide fragmentation was performed using higher energy collision-induced dissociation and normalized collision energy of 28 %. The precursor isolation window width of the quadrupole was set to 1.1 m/z. MS2 spectra were acquired with a resolution of 15,000, a fixed first mass of 100 m/z, a normalized automatic gain control target value of 100 %, and a maximum injection time of 40 ms.

2.6.4 Data analysis and visualization

Note: These steps were performed at the BayBioMS.

Peptide identification and quantification was performed using the software MaxQuant (version 1.6.3.4) with its built-in search engine Andromeda (Cox et al. 2011; Tyanova et al. 2016). MS2 spectra were searched against the *B. favorum* TMW 2.1880 proteom database (derived from genome data SAMN07674723), supplemented with common contaminants (built-in option in MaxQuant). Trypsin/P was specified as proteolytic enzyme. Carbamidomethylated cysteine was set as fixed modification. Oxidation of methionine and acetylation at the protein N-terminus was specified as variable modifications. Results were adjusted to 1 % false discovery rate (fdr) on peptide spectrum match level and protein level employing a target-decoy approach using reversed protein sequences. Label-Free Quantification (LFQ) intensities were used for protein quantification with at least 2 peptides per protein identified (Cox et al. 2011). The minimal peptide length was defined as 7 amino acids and the “match-between-runs” functionality was disabeled. Missing values were imputed by a protein-specific constant value, which was defined as the lowest detected protein-specific LFQ-value over all samples divided by two. Additionally, a maximal imputed LFQ value was defined as 15 % quantile of the protein distribution from the complete dataset. Data was visualized via omicsViewer (Meng 2022). For experiments with triplicates, t-tests were used for

statistical evaluation. Proteins were considered statistically differentially expressed, if the fdr was below 0.05 and the log10 fold change was higher than 0.30103 or below -0.30103.

2.7 Metabolomics

Metabolomics experiments were conducted to analyze the relative quantities of intracellular metabolites in different conditions. The experiments were conducted according to Weiss et al. (2022) and in cooperation with Dr. Karin Kleigrewe from the Bavarian Biomolecular Mass Spectrometry Center (BayBioMS, Freising, Germany) of the Technical University of Munich.

2.7.1 Sample Preparation

Three 10 mL cultures were used for the analysis in triplicates. The entire volume was centrifuged (8,000 × g, 8 min, 4 °C) and the supernatant discarded. The pellet was washed with 1 mL H_2O_d and centrifuged again. The pellet was then dissolved in 500 µL 70 % methanol. For cell disruption, the cells were shaken at 4 m/s for 30 s using a FastPrep-24 (MP Biomedicals Inc., Eschwege, Germany). The solution was centrifuged (10,000 × g, 10 min, 4 °C) and 300 µL of the supernatant was pipetted into a separate tube and stored at -80 °C until further analysis.

2.7.2 Analysis by mass spectrometry

Note: These steps were performed at the BayBioMS.

The untargeted analysis was performed using a Nexera UHPLC system (Shimadzu, Kyoto, Japan) coupled to a Q-TOF mass spectrometer (TripleTOF 6600, AB Sciex, Framingham, MA, USA). Separation of the samples was performed using a UPLC BEH Amide 2.1 × 100, 1.7 µm analytic column (Waters Corp., Milford, MA, USA) with a flow rate of 400 µL/min. The mobile phase was 5 mM ammonium acetate in water (eluent A) and 5 mM ammonium acetate in acetonitrile/water (95/5, v/v) (eluent B). The gradient profile was 100% B from 0 to 1.5 min, 60 % B at 8 min and 20 % B at 10 min to 11.5 min and 100 % B at 12 to 15 min. A sample volume of 5 µL was injected. The autosampler was cooled to 10 °C and the column oven heated to 40 °C. Every tenth run a quality control (QC) sample which was pooled from all samples was injected. The samples were measured in a randomized order and in Information Dependent Acquisition mode. MS settings in the positive mode were: Gas 1 55, Gas 2 65, Curtain gas 35, Temperature 500 °C, Ion Spray Voltage 5500, declustering potential 80. The mass range of the TOF MS and MS/MS scans were 50 to 2000

m/z and the collision energy was ramped from 15 to 55 V. MS settings in the negative mode were: Gas 1 55, Gas 2 65, Cur 35, Temperature 500 °C, Ion Spray Voltage - 4500, declustering potential - 80. The mass range of the TOF MS and MS/MS scans were 50–2000 m/z and the collision energy was ramped from - 15 to 55 V.

2.7.3 Data analysis and visualization

Note: These steps were performed at the BayBioMS.

The "msconvert" from ProteoWizard (Kessner et al. 2008) was used to convert raw files to mzXML (de-noised by centroid peaks). The bioconductor/R package xcms (Smith et al. 2006) was used for data processing and feature identification. More specifically, the matched filter algorithm was used to identify peaks (full width at half maximum set to 7.5 s). Then the peaks were grouped into features using the "peak density" method (Smith et al. 2006). The areas under the peaks were integrated to represent the abundance of features. The retention time was adjusted based on the peak groups presented in most of the samples. To annotate possible metabolites to identified features, the exact mass and MS2 fragmentation pattern of the measured features were compared to the records in HMBD (Wishart et al. 2018) and the public MS/MS database in MSDIAL (Tsugawa et al. 2015), referred to as MS1 and MS2 annotation, respectively. The QC samples were used to control and remove the potential batch effect, t-test was used to compare intensities. Metabolites were considered statistically differentially detected, if the fdr was below 0.05 and the log10 fold change was higher than 0.30103 or below - 0.30103. Only metabolites of category 4, i.e. with a safe annotation, were considered.

3 Results

3.1 Phylogenetic analysis of available *Bombella* whole genome data

Note: Parts of this section have already been published in Härer et al. (2022).

To investigate the evolutionary relationships within the genus *Bombella*, all available genome sequences from the NCBI database (status 02/21) and by our chair were phylogenetically analyzed. This includes genomes that were assigned to the genus *Parasaccharibacter* or *Saccharibacter* originally, but were recently identified as *Bombella* strains (Smith et al. 2021). After removing all genome with clonal origin, a total of 22 genomes were available, eight of which were assembled at our chair and corresponding strains are also available in the strain collection. These eight strains can be distinguished from the others by the designation "TMW". All strains were either isolated from the western honey bee *Apis mellifera*, the European dark bee *A. mellifera mellifera* or the red-tailed bumble bee *Bombus lapidarius*. A list of all strains, respective genome stats and origin is shown in Table 6.

Table 6: Available whole genome sequences from strains of the genus *Bombella* with respective statistics and origin. Host: 1 = *A. mellifera*, 2 = *A. mellifera mellifera and* 3 = *Bombus lapidarius*

Strain designation	Contigs	Size [mbp]	GC [%]	Host	Country
B. apis MRM1^T	7	2.03	59.59	1	KOR
B. apis ESL0368	1	1.99	59.6	1	CHE
B. apis ESL0380	10	1.98	59.57	1	CHE
B. apis ESL0387	10	2.04	59.47	1	CHE
B. apis SME1	11	2.09	59.56	1	USA
B. apis TMW 2.1882	5	2.02	59.41	1	DEU
B. apis TMW 2.1884	19	2.05	59.37	1	DEU
B. apis TMW 2.1886	39	2.05	58.86	2	AUT
B. apis TMW 2.1888	1	2.01	59.48	2	AUT
B. apis TMW 2.1890	7	2.02	59.49	1	DEU
B. apis TMW 2.1891	7	2.01	59.44	1	DEU
B. apis A29	27	2.01	59.39	1	USA
B. apis AM169	9	1.98	59.32	1	ITA
B. apis G773c	1	2.01	59.42	1	USA
B. apis 3A1	24	2.01	59.41	1	HUN
B. apis M18	11	2.08	59.35	1	HUN
B. intestini R52487^T	12	2.02	54.94	3	BEL
B. favorum TMW 2.1880^T	7	1.98	55.33	1	DEU
B. mellum TMW 2.1889^T	11	2.07	60.43	1	DEU
B. sp. ESL0378	15	1.85	52.88	1	CHE
B. sp. ESL0385	5	1.9	52.91	1	CHE
B. sp. AS1	13	1.85	52.64	1	USA

A phylogenetic tree was calculated based on whole core genome sequences (Section 2.3.4) with the closely related *Saccharibacter floricola* DSM 15669^T as the outgroup (Figure 4).

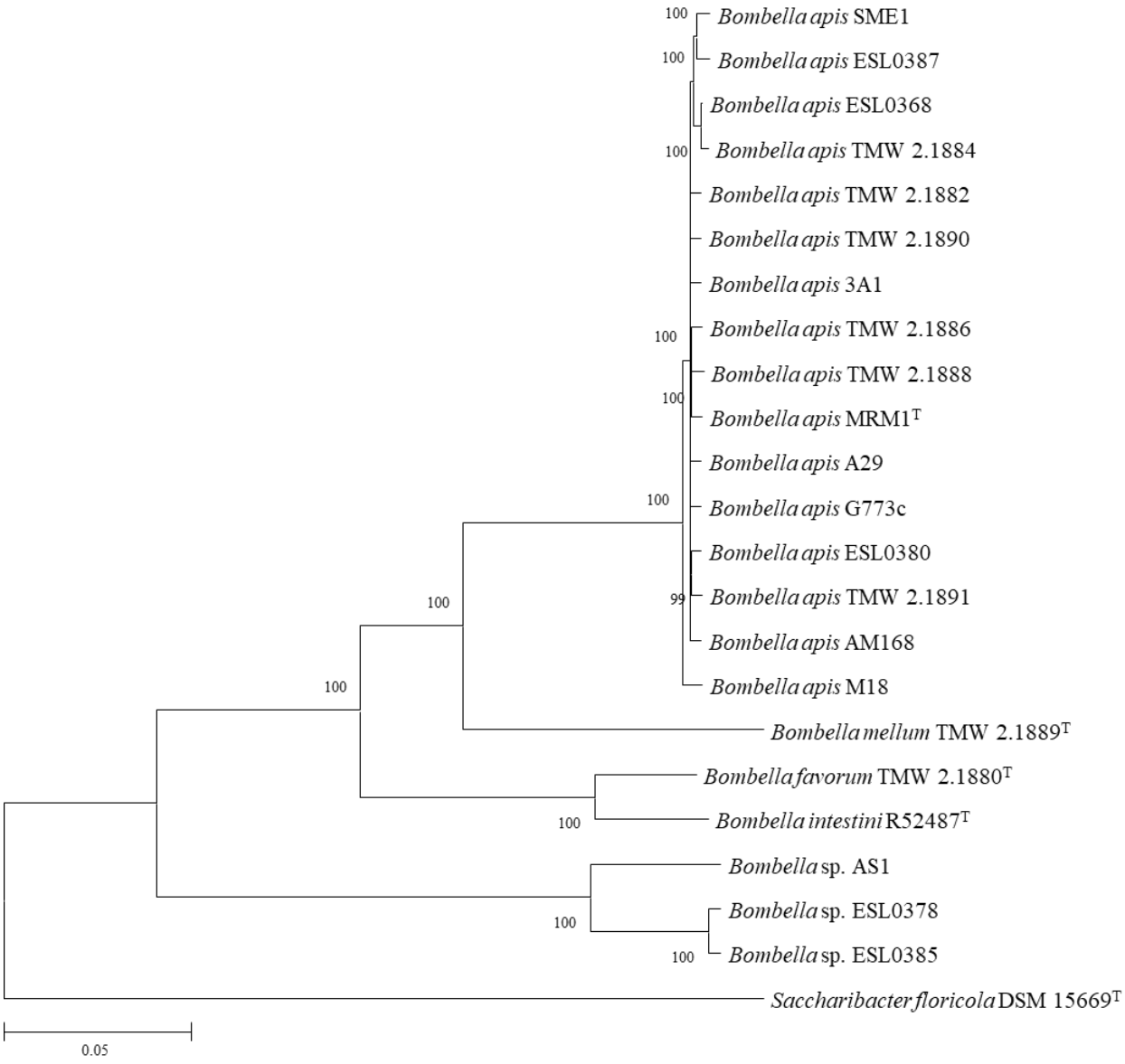


Figure 4: Phylogenetic tree of strains of the genus *Bombella* based on whole core genome sequences (1,343 genes per genome) using the Neighbor-Joining method (Saitou and Nei 1987) in MEGAX (Kumar et al. 2018). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers at nodes indicate bootstrap percentages using 1,000 replicates. *Saccharibacter floricola* DSM 15569^T (Accession: NZ_KB899333) was used as an outgroup.

The tree shows that 16 of the 22 Bombella strains belong to the *B. apis* clade. This makes it the predominant species. No strains, except for the type strains *B. favorum* TMW 2.1880^T, *B. mellum* TMW 2.1889^T and *B. intestini* R52487^T can be assigned to the other validly described species. Interestingly, the strains *Bombella* sp. AS1, *Bombella* sp. ESL0378 and *Bombella* sp. ESL0385 form their own branch, which identifies them as potential novel species. For a more detailed

phylogenetic analysis of these three strains, the OrthoANI values (section 2.3.4) towards each other and the other type strains were determined. *Bombella* sp. ESL0378 and *Bombella* sp. ESL0385 exhibit an OrthoANI value of 99.2 % making them two strains of the same species. In comparison to *Bombella* sp. AS1 they exhibit OrthoANI values below 93 %, which separates the three strains into two closely related, but different species (threshold between 94 and 96 %). Since the OrthoANI values of the three strains towards the other *Bombella* type strains are below 76 % it can be concluded that they belong to two hitherto undescribed species of the genus *Bombella*.

3.2 Diversity of the genus *Bombella* in the environment of the western honey bee

Note: Parts of this section have already been published in Härer et al. (2023b).

As described in the previous section, the genomic data of three strains from the environment of the western honey bee *Apis mellifera* could not be assigned to any validly described *Bombella* species. This is an indication that the diversity of the genus is greater than it has been described in the literature so far. This section deals with the isolation and characterization of novel strains in order to obtain a better coverage and new insights into the genus *Bombella*.

3.2.1 Isolation and taxonomic assignment of novel *Bombella* strains

To gain a better understanding of the diversity of the genus *Bombella*, various biological samples from the environment of the western honey bee *Apis mellifera* were analyzed for the presence of *Bombella* strains. The aim was to isolate strains that did not belong to the genus *B. apis*, since this species was already strongly represented in the literature and in our own strain collection. Samples were taken with inoculation loops from four different beehives in Freising (Bavaria, Germany) and Plüderhausen (Baden-Württemberg, Germany). Various niches were examined, such as drone brood, honeycombs and royal jelly. Dead bees were also collected and analyzed. Samples were diluted in 1 mL saline (0.9 % (w/v) NaCl) and plated out LMG 404 agar plates.

After two days at 30 °C, yellowish colonies with diameter between 0.5 and 2 mm were subjected to MALDI-TOF MS analysis (Section 2.0) for species assignment. Several hundred colonies were analyzed. Samples that were assigned to other genera or identified as *B. apis* with scores over 2.3 (highly probable species identification) were not considered. Strains with scores below 2.3 (secure genus identification, probable species identification) were further investigated. A total of 32 potentially novel *Bombella* strains were identified.

Next, the 16S rRNA gene was partially amplified and sequenced using the standard primers 27F and 1507R (688 bp; Section 2.1.3). For strains with similar MALDI-TOF MS spectra, only single representative strains from the corresponding groups were sequenced. By evaluating all known partial 16S rRNA sequences of the genus, a species differentiation threshold of 99.85 % identity was applied. With this threshold 23 of the 32 candidates could be assigned to *B. apis*. In addition, five candidates were assigned to the genus *Pseudomonas*. The remaining four candidates *Bombella* sp. TMW 2.2543, *Bombella* sp. TMW 2.2556, *Bombella* sp. TMW 2.2558 and *Bombella* sp. TMW 2.2559 could be assigned to the genus *Bombella*, but not to any validly described species.

3.2.2 Phylogenetic analysis of four *Bombella* strains without taxonomic affiliation

The genomic DNA of *Bombella* sp. TMW 2.2543, *Bombella* sp. TMW 2.2556, *Bombella* sp. TMW 2.2558 and *Bombella* sp. TMW 2.2559 was purified and externally sequenced (Section 2.3.2). The statistics of the assembled genomes with respective NCBI accession numbers are summarized in Table 7.

Table 7: Genome data characteristics of the four unassigned *Bombella* strains.

***Bombella* sp.**	**Size [mbp]**	**Contigs**	**GC [%]**	**Coverage**	**CDS**	**NCBI Genome Accession**
TMW 2.2543	2.01	14	56.2	591x	1,766	JANIDY000000000
TMW 2.2556	1.88	27	53.0	607x	1,715	JANIDX000000000
TMW 2.2558	1.91	17	52.7	536x	1,740	JANIDW000000000
TMW 2.2559	1.87	27	58.2	523x	1,699	JANIDV000000000

To assess whether the four strains can be assigned to described *Bombella* species or are strains of novel undescribed species, OrthoANI and isDDH values were calculated (Section 2.3.4) towards the type strains and towards each other (Table 8). The species demarcation threshold for OrthoANI values lies below 95 % and below 60 % for isDDH values. Since all values, even among themselves, are below these thresholds, the strains can be considered as type strains of novel species of the genus *Bombella*: *B. pluederhausensis* sp. nov. TMW 2.2543^T (= DSM 114872^T, = LMG 32791^T), *B. pollinis* sp. nov. TMW 2.2556^T (= DSM 114874^T, = LMG 32792^T), *B. saccharophila* sp. nov. TMW 2.2558^T (= DSM 114877^T, = LMG 32793^T) and *B. dulcis* sp. nov. TMW 2.2559^T (= DSM 114877^T, = LMG 32794^T).

Table 8: isDDH and orthoANI values between the four novel strains and described *Bombella* species. Strains: (1) *B. intestini* R52487^T, (2) *B. favorum* TMW 2.1880^T, (3) *B. mellum* TMW 2.1889^T, (4) *B. apis* MRM1^T, (5) *Bombella sp.* TMW 2.2543^T, (6) *Bombella sp.* TMW 2.2556^T, (7) *Bombella sp.* TMW 2.2558^T, (8) *Bombella sp.* TMW 2.2559^T.

		orthoANI							
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
	1	/	94.3	81.0	82.4	80.9	75.2	75.0	81.8
	2	56.3	/	80.9	82.6	81.2	75.2	75.3	81.8
	3	22.7	22.9	/	85.7	82.2	74.3	74.3	85.4
isDDH	**4**	24.4	24.7	29.5	/	81.7	74.8	74.6	83.3
	5	23.2	23.3	24.6	23.9	/	75.2	75.0	83.4
	6	18.9	18.9	18.5	18.5	19.0	/	92.8	74.9
	7	18.9	18.9	18.5	18.8	18.9	48.4	/	74.9
	8	23.8	24.1	29.1	25.7	26.2	18.7	18.6	/

To visualize the phylogenetic relationship between the novel and the described type strains, as well as the previously unassigned strains, a phylogenetic tree based on whole core genome sequences is shown in Figure 5 (Section 2.3.4).

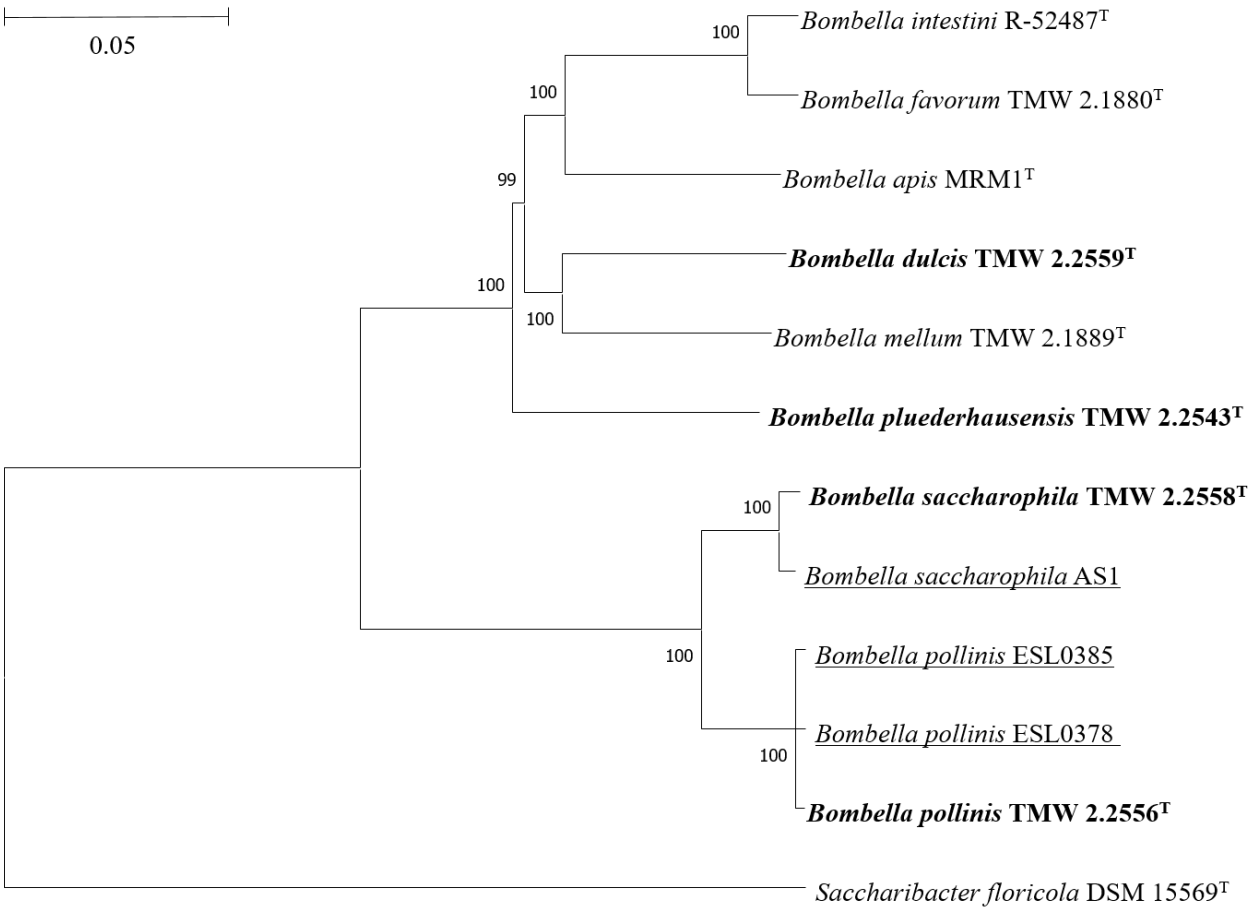


Figure 5: Phylogenetic tree of novel (bold), previous unassigned (underlined) and described *Bombella* species based on whole core genome sequences (1,279 genes per genome) using the Neighbor-Joining method (Saitou and Nei 1987) in MEGAX (Kumar et al. 2018). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Numbers at nodes indicate bootstrap percentages using 1,000 replicates. *Saccharibacter floricola* DSM 15569^T (Accession: NZ_KB899333) was used as an outgroup.

The phylogenetic tree shows that *B. pluederhausensis* TMW 2.2543 and *B. dulcis* TMW 2.2559 form single branches between the other described species. *B. saccharophila* TMW 2.2558 and *B. pollinis* TMW 2.2556, on the other hand, form a clade of their own, indicating a more distant evolutionary relationship to the other type strains.

The three strains *Bombella* sp. ESL0378, *Bombella* sp. ESL0385 and *Bombella* sp. AS1, which were previously discussed as *Bombella* strains without species affiliation, can also be assigned to this clade. *B. pollinis* TMW 2.2556 exhibits orthoANI values of 99.35 % towards *Bombella* sp. ESL0378 and 99.27 % towards *Bombella* sp. ESL0385. Corresponding isDDH values of *B. pollinis* sp. nov. TMW 2.2556^T are 93.4 % in relation to *Bombella* sp. ESL0378 and 92.9 % in relation to *Bombella* sp. ESL0385. Moreover, the orthoANI and isDDH values of *B. saccharophila* sp. nov.

TMW 2.2558^T in relation to *Bombella* sp. AS1 are 99.07 % and 91.6 %, respectively. All values are above the species demarcation, assigning the corresponding strains to the novel species.

3.2.3 Physiological characterization of novel *Bombella* species

In order to test whether the novel type strains *Bombella sp.* TMW 2.2543^T, *Bombella sp.* TMW 2.2556^T, *Bombella sp.* TMW 2.2558^T and *Bombella sp.* TMW 2.2559^T have unique physiological properties, various experiments were performed with the known type strains *B. apis* MRM1^T, *B. intestini* R52487^T, *B. favorum* TMW 2.1880^T and *B. mellum* TMW 2.1889^T as comparison (Section 2.1.4).

All type strains were tested for motility, dihydroxyacetone formation from glycerol, pellicle formation, acetate production from ethanol and assimilation of ammonium nitrogen. In addition, experiments were done to determine at which temperatures and pH values growth still occurs and where the corresponding optimum lies. For NaCl and glucose, the maximum concentrations at which growth was still measurable were determined. The results are summarized in Table 9.

All type strains are mesophilic and have an optimum growth temperature at approx. 30 °C. They also grow in acidic environments up to a pH of 3.5, but the optimal pH is in the neutral range between 6 and 9. All strains are extremely glucose tolerant (up to 550 g/L glucose), but sensitive to NaCl (max. 15 g/L). In addition, all type strains form pellicles, but none are capable of acetate production from ethanol or ammonium nitrogen assimilation. Differences between strains were found in motility and the formation of dihydroxyacetone from glycerol.

Table 9: Physiological similarities and differences between all proposed and described *Bombella* species. Strains: (1) *B. intestini* R52487^T, (2) *B. favorum* TMW 2.1880^T, (3) *B. mellum* TMW 2.1889^T, (4) *B. apis* MRM1^T, (5) *B. pluederhausensis* TMW 2.2543, (6) *B. pollinis* TMW 2.2556^T, (7) *B. saccharophila* TMW 2.2558^T, (8) *B. dulcis* TMW 2.2559^T.

Characteristic	1	2	3	4	5	6	7	8
Motility	–	–	+	–	+	+	–	+
Temperature range [°C]	25 – 35	20 – 35	20 – 35	20 – 35	20 – 40	25 – 35	25 – 35	25 – 35
Temperature optimum [°C]	30 – 35	30	30 – 35	30 – 35	25 – 35	30 – 35	30 – 35	30
pH range	3.5 – 9	3.5 – 9	3.5 – 9	3.5 – 9	3.5 – 9	3.5 – 9	3.5 – 9	3.5 – 9
pH optimum	7 – 9	6 – 8	7 – 9	5 – 8	6 – 8	7 – 9	7 – 9	6 – 8
Growth with 500 g/L glucose	w	w	w	w	w	w	w	–
Growth with 5 g/L NaCl	w	+	+	+	+	w	+	w
Dihydroxyacetone from glycerol	+	+	–	–	+	–	+	w
Pellicle formation	+	+	+	+	+	+	+	+
Acetate production from ethanol	–	–	–	–	–	–	–	–
Assimilation of ammonium nitrogen	–	–	–	–	–	–	–	–

3.3 Comparative genomics within the genus *Bombella*

Note: Parts of this section have already been published in Härer et al. (2022).

After all genomes of the genus *Bombella* could be assigned to a validly described species, various comparative genomics tools were used to identify intra- and interspecies differences. Both the already sequenced genomes from section 3.1 and the genomes of the novel species from section 3.2 were considered.

3.3.1 Main cytoplasmic carbohydrate metabolism

All open reading frames (ORFs) of the genomes were translated into the corresponding amino acid sequences and grouped by using the BADGE tool (Section 2.3.3). Each group represents highly homologous proteins that likely have the same or similar cellular functions. This made it possible to determine whether the corresponding genes were present in all genomes or only in individual genomes.

The Embden–Meyerhof–Parnas (EMP) pathway, the tricarboxylic acid (TCA) cycle and the pentose phosphate pathway were reconstructed (Figure 6) based on the works from Li et al. (2016)

and Prust et al. (2005). Locus tags are summarized in Supplementary Table S1. All analyzed *Bombella* genomes lack a phospho-fructokinase gene, making the EMP pathway incomplete. The presence of a fructose-1,6-bisphosphatase gene (enzyme 3) should allow all *Bombella* strains to perform gluconeogenesis. All genes encoding the enzymes of the pentose phosphate pathway were identified. Genes encoding 6-phosphogluconate dehydratase and 2-dehydro-3-deoxyphosphogluconate aldolase complete the Entner-Doudoroff (ED) pathway. No genes encoding succinyl-CoA synthetase, succinate dehydrogenase or malate dehydrogenase were identified, suggesting an incomplete TCA cycle. All strains of the species *Bombella mellum*, *B. saccharophila* and *B. pollinis* also lack genes for the enzymes citrate synthase, aconitate hydratase and isocitrate dehydrogenase. Genes for the incorporation of D-fructose into intracellular carbohydrate metabolism were identified and confirmed in growth experiments with available strains. In addition, a gene encoding an extracellular enzyme with potential invertase activity (GH 32 family) was identified that should allow all strains *Bombella* to grow on sucrose (Supplementary Table S2). To confirm the *in silico* results, the growth of available *Bombella* spp. ("TMW" designation) was verified on agar plates with 50 g/L D-fructose or sucrose as the main carbon source. Growth was observed after 48 h for all tested strains.

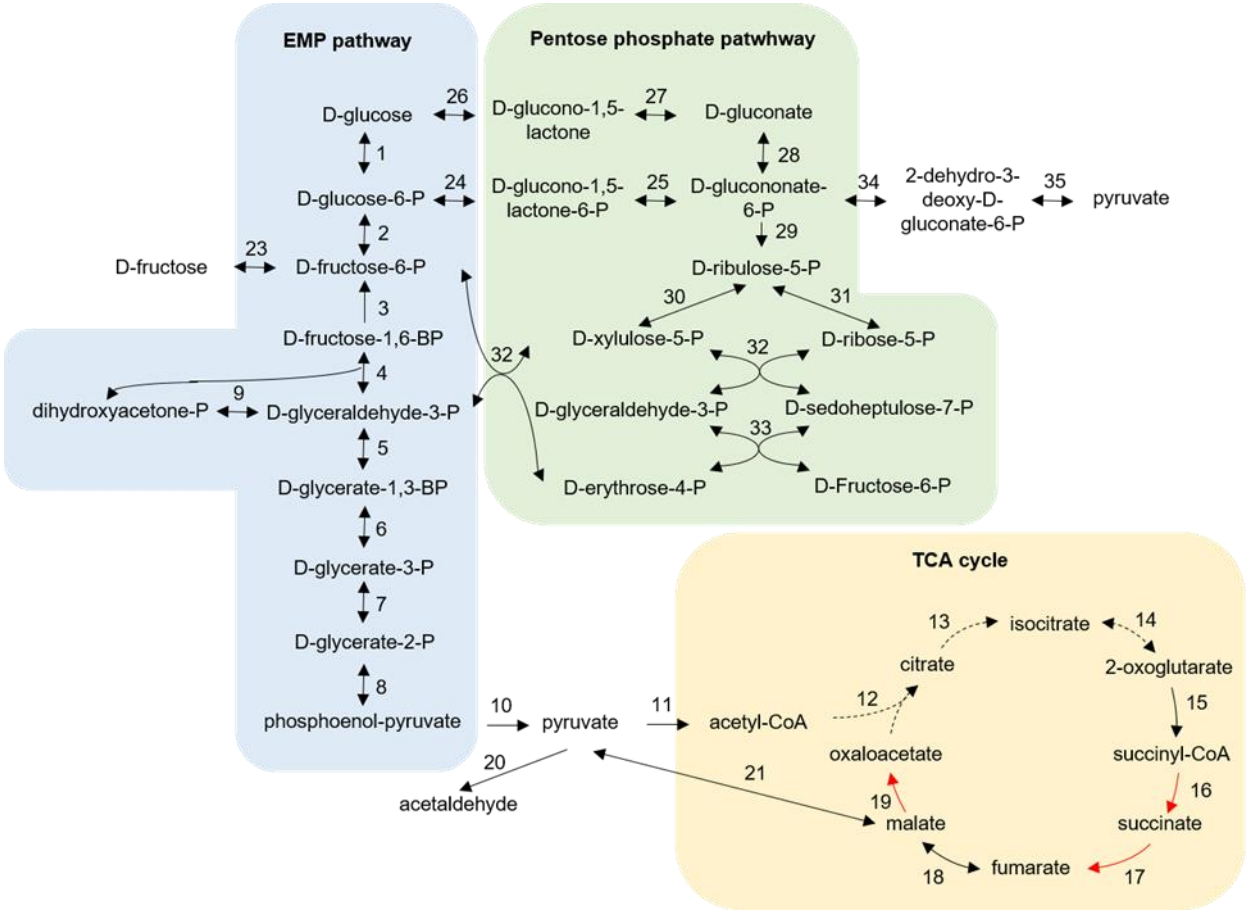


Figure 6: Predicted cytoplasmic carbohydrate metabolism of *Bombella* spp.. Dashed arrows indicate genes not present in *B. mellum*, *B. saccharophila* and *B. pollinis* genomes. Red arrows indicate missing genes in all genomes. 1: glucokinase; 2: glucose-6-phosphate isomerase; 3: fructose-1,6-bisphosphatase; 4: fructose-bisphosphate aldolase; 5: glyceraldehyde-3-phosphate dehydrogenase; 6: phosphoglycerate kinase; 7: phosphoglycerate mutase; 8: enolase; 9: triose-phosphate isomerase; 10: pyruvate kinase; 11: pyruvate dehydrogenase complex; 12: citrate synthase; 13: aconitate hydratase; 14: isocitrate dehydrogenase; 15: oxoglutarate dehydrogenase complex; 16: succinyl-CoA synthetase; 17: succinate dehydrogenase; 18: fumarase; 19: malate dehydrogenase; 20: pyruvate decarboxylase; 21: malate dehydrogenase (oxaloacetate-decarboxylating); 23: fructokinase; 24: glucose-6-phosphate dehydrogenase; 25: 6-phosphogluconolactonase; 26: glucose 1-dehydrogenase; 27: gluconolactonase; 28: gluconoate kinase; 29: phosphogluconate dehydrogenase; 30: ribulose-phosphate 3-epimerase; 31: ribose-5-phosphate isomerase; 32: transketolase; 33: transaldolase; 34: 6-phosphogluconate dehydratase; 35: 2-dehydro-3-deoxyphosphogluconate aldolase.

3.3.2 Membrane-bound dehydrogenases and respiratory enzymes

Four membrane-bound DHs were identified in all analyzed *Bombella* genomes. A PQQ-dependent glucose DH and a gluconate-2 DH catalyze the oxidation of glucose to gluconate to 2-ketogluconate. In addition, all strains contained a quinone-dependent dihydroorotate DH and a D-lactate DH. Further, an alcohol DH (ALDH) with an unknown substrate spectrum was identified.

The corresponding genes are present twice in each genome, except in strains of the species *B. pollinis* or *B. saccharophila*, where single, truncated versions were identified. The respiratory chain of *Bombella* spp. consists of an NADH DH (type II), a bo3-type cytochrome c oxidase, a cytochrome bc1 complex, a flavoprotein-ubiquinone oxidoreductase and a cytochrome D ubiquinol oxidase. The only exception is *B. intestini* R52487^T, where no cytochrome D ubiquinol oxidase was identified.

3.3.3 Tetracycline resistance genes

Two genes associated with resistance to tetracycline antibiotics were identified in the *Bombella apis* MRM1^T genome: a tetracycline resistance transcriptional repressor (tetR; IGM82_03615) and a tetracycline efflux major facilitator superfamily (MFS) transporter (tetG; IGM82_03620). No other genes associated with tetracycline resistance were identified in any of the other genomes. In order to confirm the resistance, available strains were tested for resistance toward tetracycline (30 µg) and doxycycline (30 µg) via a disk diffusion method (Section 2.1.5). Growth inhibition zones varied between 27 and 36 mm for tetracycline and from 23 to 31 mm for doxycycline. No growth inhibition was measured for *Bombella apis* MRM1^T, confirming the *in silico* finding of the resistance genes tetR and tetG.

3.3.4 Antifungal metabolite gene cluster

Recently, a type 1 polyketide synthase (T1PKS) gene cluster was associated with potential antifungal properties in *B. apis* (Miller et al. 2021). To check whether this feature was present in all *Bombella* genomes the antiSMASH webtool was used (Section 2.3.3). The gene cluster was identified in all *Bombella* genomes. This could indicate that antifungal properties are distributed throughout the genus.

To test whether this is a unique property of *Bombella* species, additional AAB genomes were analyzed. The T1PKS gene cluster was identified in all tested AAB species genomes, including *Saccharibacter floricola* DSM 15669^T (Accession: NZ_KB899333), *Gluconacetobacter diazotrophicus* PA1 5 (Accession: CP001189) and *Asaia bogorensis* NBRC 16594 (Accession: NZ_AP014690) (Supplementary Table S3).

3.4 Glucose tolerance of *Bombella* spp.

All but one type strain of the genus *Bombella* can tolerate glucose concentrations of 500 g/L (Section 3.2.3). For a more detailed analysis, the glucose tolerance of all available strains was investigated by monitoring of the growth at five different glucose concentrations from 100 to 500 g/L. The experiments were performed in 250 µL scale in a plate reader. The maximum growth rates [h^{-1}] were calculated for each glucose concentration and strain (Section 2.1.2). The growth curves of *Bombella mellum* TMW 2.1889 and *Bombella pollinis* TMW 2.2556 could not be evaluated because both strains tend to form pellicles, which affected OD measurements. For comparison purposes, *Gluconobacter oxydans* DSM46615 was also tested. The results are shown in Figure 7.

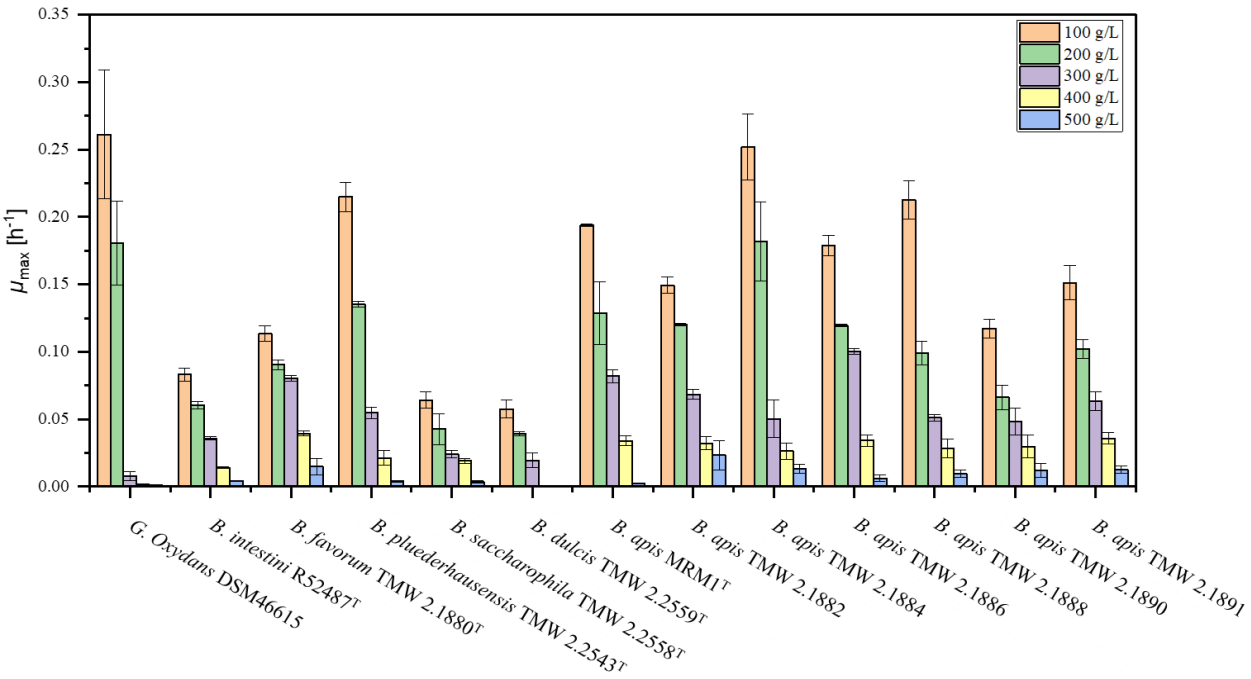


Figure 7: Maximum growth rates μ_{max} of *Bombella* strains from six different species at 100, 200, 300, 400 or 500 g/L glucose and 30 °C. *G. oxydans* DSM46615 was used as a comparison. Cultures were grown in microtiter scale (250 µL).

G. oxydans DSM46615 showed comparatively high maximum growth rates at 100 and 200 g/L glucose (above 0.15 h^{-1}). However, at 300 g/L glucose, a reduction in the maximum growth rate below 0.01 h^{-1} was calculated. For 400 and 500 g/L glucose, no measurable growth was detected. The 12 *Bombella* strains tested mostly had lower maximum growth rates at 100 and 200 g/L glucose in comparison to *G. oxydans* DSM46615. However, at 400 g/L glucose, growth rates between 0.014

and 0.039 h^{-1} could be calculated. Even at 500 g/L, maximum growth rates above 0.01 h^{-1} could still be calculated for five strains. The exception was *B. dulcis* TMW 2.2559 which showed relatively low maximum growth rates at 100, 200 and 300 g/L glucose and no growth at 400 and 500 g/L glucose.

It can be concluded that 11 out of 12 *Bombella* strains showed a higher tolerance towards glucose than *G. oxydans* DSM46615. With one exception, the strains showed growth up to 400 g/L, reaching their growth limits at higher concentrations.

3.5 NaCl tolerance of *Bombella favorum* TMW 2.1880

As shown in the previous section, *Bombella* strains generally have a tolerance to high glucose concentrations. However, as shown in section 3.2.3, all type strains are sensitive to NaCl, with concentrations as low as 5 g/L inhibiting growth. In this section, the inhibitory effect of NaCl on the growth of *B. favorum* TMW 2.1880 was investigated and compared to the inhibitory effect of 400 g/L glucose. *B. favorum* TMW 2.1880 was selected because in previous experiments high tolerance towards glucose, no formation of pellicles under shaking, and uniform growth was observed. Growth experiments were carried out with NaCl concentrations ranging from 0 to 15 g/L. The base medium was LMG 404 (50 g/L glucose and 10 g/L yeast extract), which was supplemented with NaCl. Glucose stress medium contained 400 g/L glucose. Growth curves inoculated with the same overnight culture are shown in Figure 8.

Growth of *B. favorum* TMW 2.1880 was observed in media containing 5 and 10 g/L NaCl. At 15 g/L NaCl growth was completely inhibited. Compared to glucose stress, NaCl growth curves have shorter lag phases but reach lower maximum optical densities. For subsequent experiments, a concentration of NaCl must be determined that will stress the cells similarly to 400 g/L glucose. Based on the growth curves, this stress level was set at 10 g/L NaCl. These concentrations correspond to 2.2 M glucose and 0.12 M NaCl. It has to be considered that in the solubilized state this corresponds to 0.12 M Na^+ ions and 0.12 M Cl^- ions, i.e. twice the number of dissolved molecules.

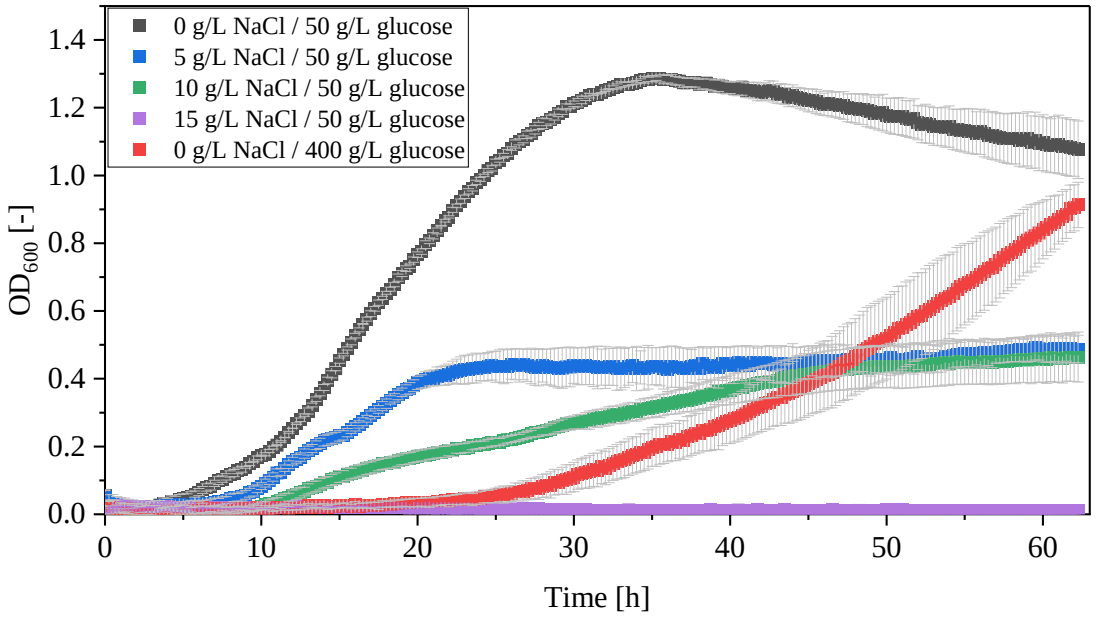


Figure 8: Growth curves of *B. favorum* TMW 2.1880 in media with NaCl concentrations between 0 and 15 g/L or 400 g/L glucose at 30 °C. Cultures were grown in microtiter scale (250 µL).

3.6 Cellular stress response of *B. favorum* TMW 2.1880 to glucose and NaCl

A proteomic analysis was performed to investigate the cellular stress response of *B. favorum* TMW 2.1880 to glucose and NaCl stress (Section 2.6). The concentrations in which the growth of the cells are similarly inhibited were defined in the previous sections and set to 400 g/L glucose and 10 g/L NaCl. The experiment was performed in singletons due to the high number of data points for the proteomic analysis, which did not allow statistical evaluation. An over-night culture of *B. favorum* TMW 2.1880 (OD = 0.30) was used to inoculate 1 L of each stress media. LMG 404 medium (50 g/L glucose, no NaCl) was used as the unstressed reference medium. The cultures were incubated in 3 L baffled shaking flasks at 30 °C and 50 rpm. Growth was monitored via the OD. For each OD measurement, samples were also collected for proteomic analysis. Thus, the expression level of all detectable proteins could be measured over the entire cultivation period of 27 h. As shown before in smaller scale (Section 3.5), glucose stress prolongs the lag phase, while NaCl stress reduces the maximum OD. However, the stress level on the cells appears to be comparable (Figure 9). The LC/MS measurements, data processing and quality check of the proteomics data were performed externally. In total, 1,508 proteins were detectable out of 1,707 theoretically possible coding sequences.

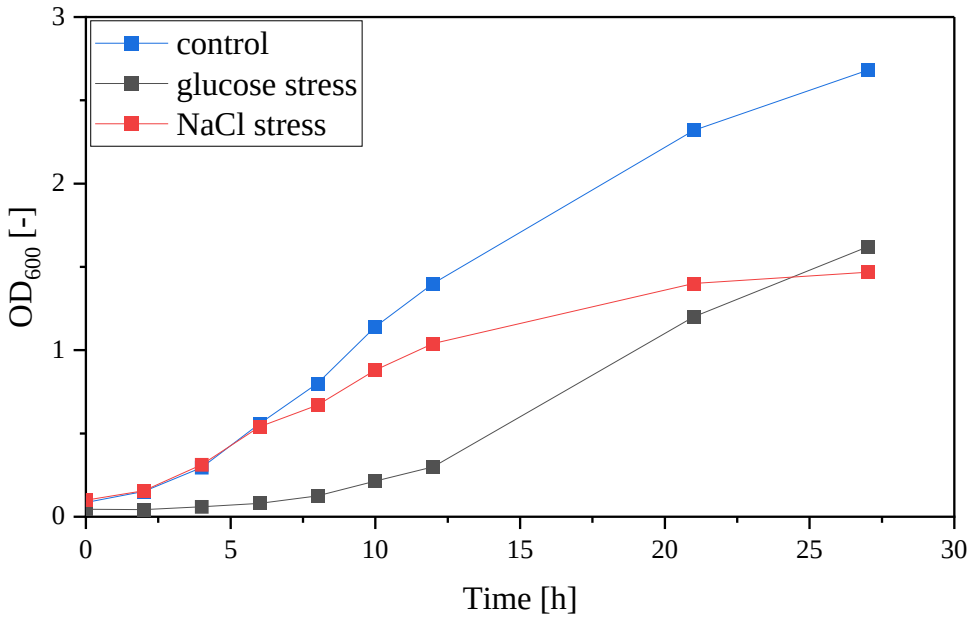


Figure 9: Growth curves of *B. favorum* TMW 2.1880 in media with 400 g/L glucose (glucose stress) or 10 g/L NaCl (NaCl stress) in comparison to unstressed reference medium (no stress; 50 g/L glucose, no NaCl). Each measurement point represents a sample collection for proteomic analysis. Starting volume of the cultures was 1 L each. Incubation was done at 30 °C and 50 rpm.

The proteomic data was analyzed to identify proteins that were differentially expressed by *B. favorum* TMW 2.1880 under glucose or NaCl stress. Because the cells were washed before sample preparation, the secreted proteins could not be considered. All proteins were named according to the NCBI locus tags. The locus tags are numbered in increments of 5 according to their position in the genome. The InterPro web tool was used to assign cellular functions on the amino acid sequences (Section 2.3.3).

3.6.1 Transporter associated protein expression under glucose or NaCl stress

Nine transporter associated proteins were identified that were differentially expressed under NaCl or glucose stress (Figure 10). Expression of the sugar transporter CPA57_01025 (A) was decreased under glucose stress compared to NaCl stress or in the control medium. The ABC transporter associated protein CPA57_02560 (B) showed increasing expression over the course of cultivation under NaCL stress and in the control medium, while the expression level remained constant under glucose stress. Expression of the protein CPA57_07545 (C), annotated as an alginate exporter, decreased in all media, but most under NaCl stress.

In the three different media, the expression courses of CPA_07315 (D), CPA_07320 (E) and CPA_07325 (F) were very similar: a decrease was detected in the first five hours of cultivation,

after which expression plateaued at a comparatively increased level under NaCl stress and a decreased level under glucose stress. It is very likely that the three proteins are subunits of an efflux transporter. This is also supported by the fact that the locus tags are consecutive, i.e. the corresponding genes are present in the genome in a successive order.

The multidrug efflux transporter-associated protein CPA_07700 (G) showed increased expression under glucose stress compared to expression under NaCl stress or in control medium. CPA_07835 (H) and CPA_07840 (I) are most likely two units of an ABC transporter that is more expressed under NaCl stress and less expressed under glucose stress compared to the control medium.

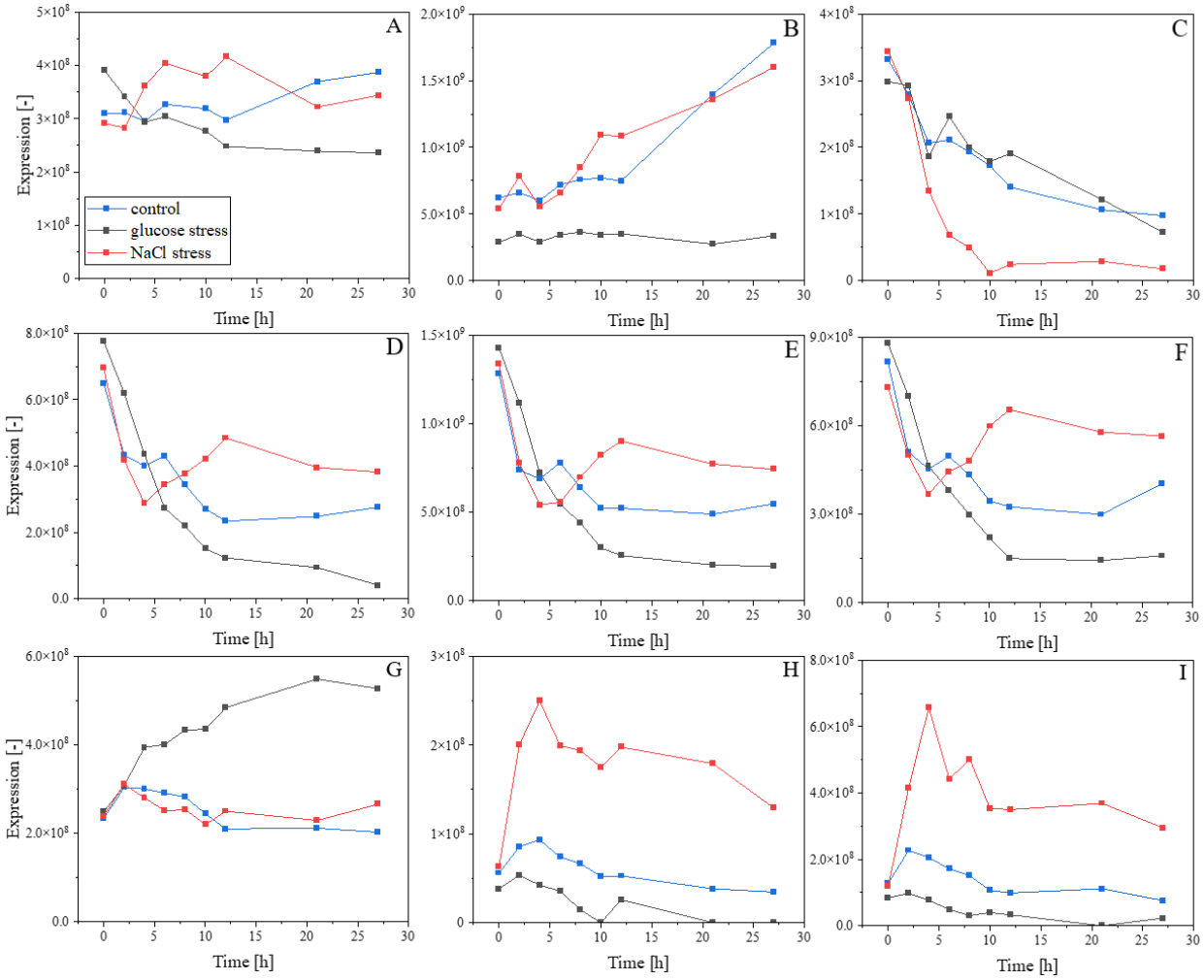


Figure 10: Relative expression levels of transporter associated proteins CPA57_01025 (A), CPA57_02560 (B), CPA57_07545 (C), CPA_07315 (D), CPA_07320 (E), CPA_07325 (F), CPA_07700 (G), CPA_07835 (H) and CPA_07840 (I) in *B. favorum* TMW 2.1880 under glucose or NaCl stress.

From this it can be concluded that there is no clear tendency for an increase or decrease in the expression of transporter-associated proteins under cell stress due to high molecular concentrations in the extracellular space. The stressor molecule (glucose or NaCl) also has an influence on which proteins are expressed higher or lower.

3.6.2 Upregulated proteins under glucose stress

Three enzymes with potential oxidoreductase activity showed increased expression under glucose stress (Figure 11): CPA57_07465, CPA57_07370 and CPA57_07345. None of the proteins contain any motifs that are indicative of a membrane-bound localization. The sequence of CPA57_07465 contains both an ALDH like C- and N-terminal sequence, but no specific catalyzed reaction could be predicted. CPA57_07370 was annotated as mannitol DH, since both the corresponding C- and N-terminal sequences were identified. CPA57_07345 was also identified as an alcohol DH, more specific as butanol DH-like.

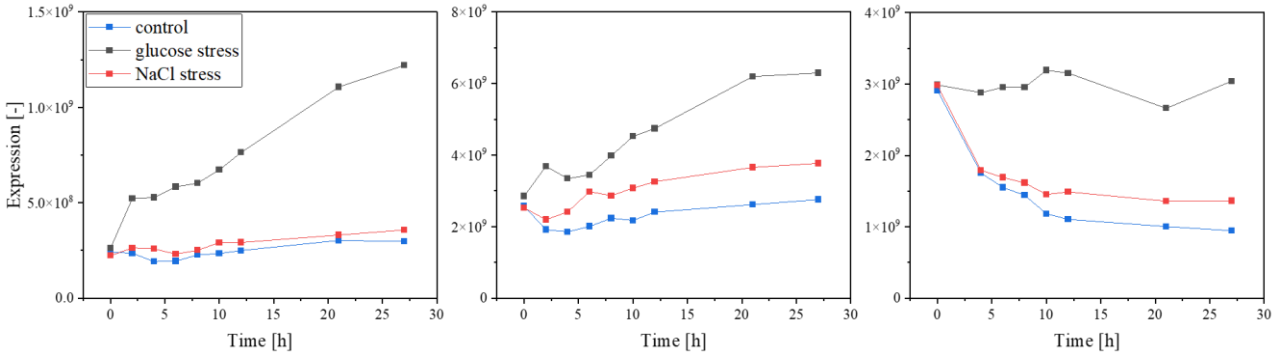


Figure 11: Relative expression levels potentially oxidoreductase active enzymes CPA57_07465 (left), CPA57_07370 (middle) and CPA57_07345 (right) in *B. favorum* TMW 2.1880 under glucose or NaCl stress.

Two other proteins that showed comparatively higher expression under glucose stress are CPA57_08255 and CPA57_04705 (Figure 12). CPA57_08255 is annotated as a pyrroline-5-carboxylate reductase, a key enzyme of the proline metabolism, CPA57_04705 as a molecular chaperone.

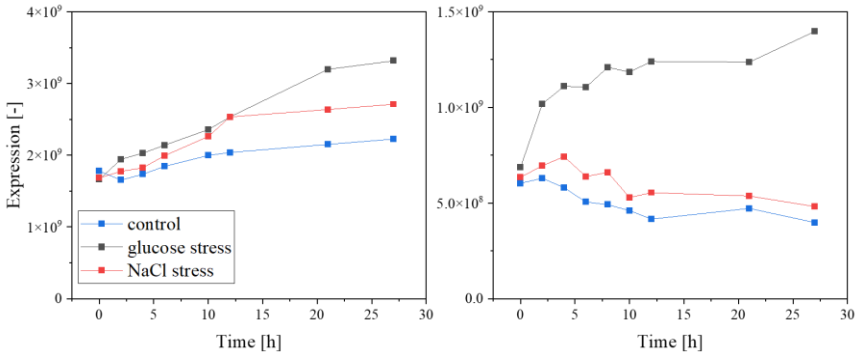


Figure 12: Relative expression levels of CPA57_08255 (left) and CPA57_04705 (right) in *B. favorum* TMW 2.1880 under glucose or NaCl stress.

3.6.3 Differentially expressed proteins under NaCl stress

The expression of nine proteins that are most likely single units of a complex Type IV secretion system (T4SS) was induced under NaCl stress (Figure 13). The proteins are CPA57_00025 (A), CPA57_00040 (B), CPA57_00045 (C), CPA57_00050 (D), CPA57_00055 (E), CPA57_00060 (F), CPA57_00065 (G) and CPA_00070 (H). Annotations from the "NCBI Prokaryotic Genome Annotation Pipeline" (PGAP) and from the InterPro webserver of the upregulated proteins and other proteins that are associated with the T4SS are shown in Table 10. None of the nine proteins were detected at the beginning of the cultivation, but were already detectable after 2 h in the medium containing 10 g/L NaCl. After that a relatively constant expression level was observed until the end of the experiment.

On a genomic level, the ORFs of the proteins CPA57_00030 and CPA57_00035 are located between the genes of the nine proteins. CPA57_00035, annotated as Plasmid conjugal transfer TrbL/VirB6, was not detected in any sample, so it is possibly located extracellularly or the quantity is below the detection limit. CPA57_00030, annotated as VirB5, was only detected in the presence of NaCl stress, but only at the 4, 12 and 27 h samples and with very low quantities. In this case, the quantity is probably at the lower limit and is therefore not detected in some samples. CPA57_00010 (VirB1), CPA57_00015 (VirB2) and CPA57_00020 (VirB3) are also associated with T4SS, but could not be detected in any sample.

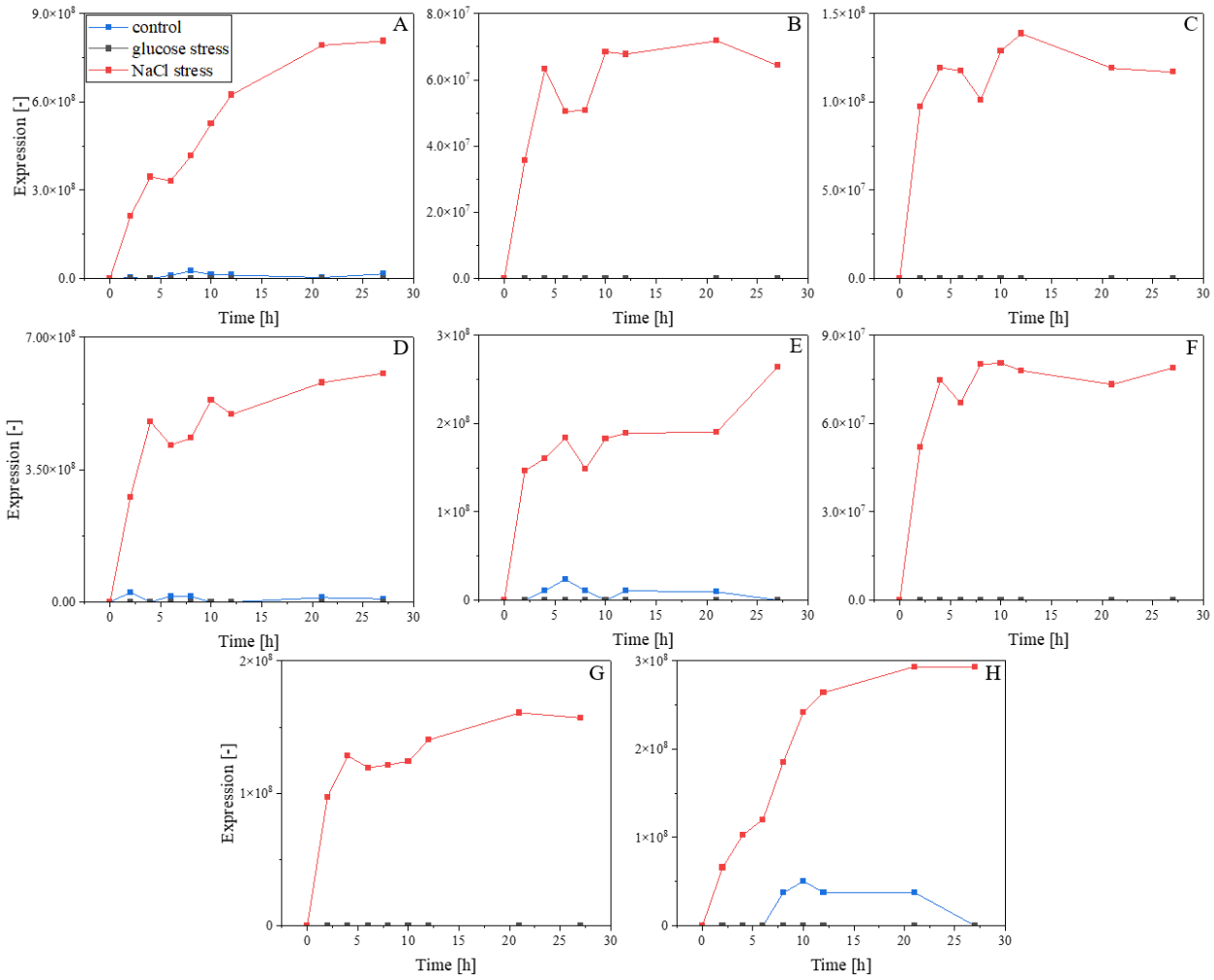


Figure 13: Relative expression levels of T4SS associated proteins (A) CPA57_00025, (B) CPA57_00040, (C) CPA57_00045, (D) CPA57_00050, (E) CPA57_00055, (F) CPA57_00060, (G) CPA57_00065 and (H) CPA_00070 in *B. favorum* TMW 2.1880 under glucose or NaCl stress.

Table 10: PGAP and InterPro annotations of *B. favorum* TMW 2.1880 Type IV secretion system associated proteins. Proteins with bold locus tags were up-regulated under NaCl stress.

Locus Tag	PGAP annotation	InterPro annotation
CPA57_00010	T4SS VirB1	None predicted
CPA57_00015	T4SS VirB2	Conjugal transfer TrbC/type IV secretion VirB2
CPA57_00020	T4SS VirB3	T4SS, VirB3 / TrbD / AvhB
CPA57_00025	T4SS VirB4	CagE, TrbE, VirB component of T4SS
CPA57_00030	transporter	T4SS, VirB5
CPA57_00035	conjugal transfer protein TraH	Plasmid conjugal transfer TrbL/VirB6
CPA57_00040	T4SS VirB7	Outer membrane lipoprotein virB7
CPA57_00045	T4SS VirB8	None predicted
CPA57_00050	T4SS VirB9	Conjugal transfer, TrbG/VirB9/CagX
CPA57_00055	type VI secretion protein	T4SS, VirB10/PtlG
CPA57_00060	P-type DNA transfer ATPase VirB11	T4SS protein VirB11
CPA57_00065	conjugal transfer protein TraG	T4SS protein TraG / VirD4
CPA57_00070	hypothetical protein	None predicted

Besides the T4SS operon, other proteins were identified whose genes appear to be grouped in an operon and were less expressed under NaCl stress. The operon consists of 75 ORFs: CPA57_06070 to CPA57_06440. Since not all data can be shown, the relative expression of six exemplary proteins whose genes are distributed throughout the operon are shown in Figure 14. Out of the 75 proteins encoded on the operon, 33 were directly linked to cell motility by the EggNOG-Mapper (Section 2.3.3), another nine to signal transduction mechanisms. The operon most likely contains all the genetic material for flagellar proteins and their regulatory mechanisms. NaCl stress on *B. favorum* TMW 2.1880 thus appears to result in reduced cell motility.

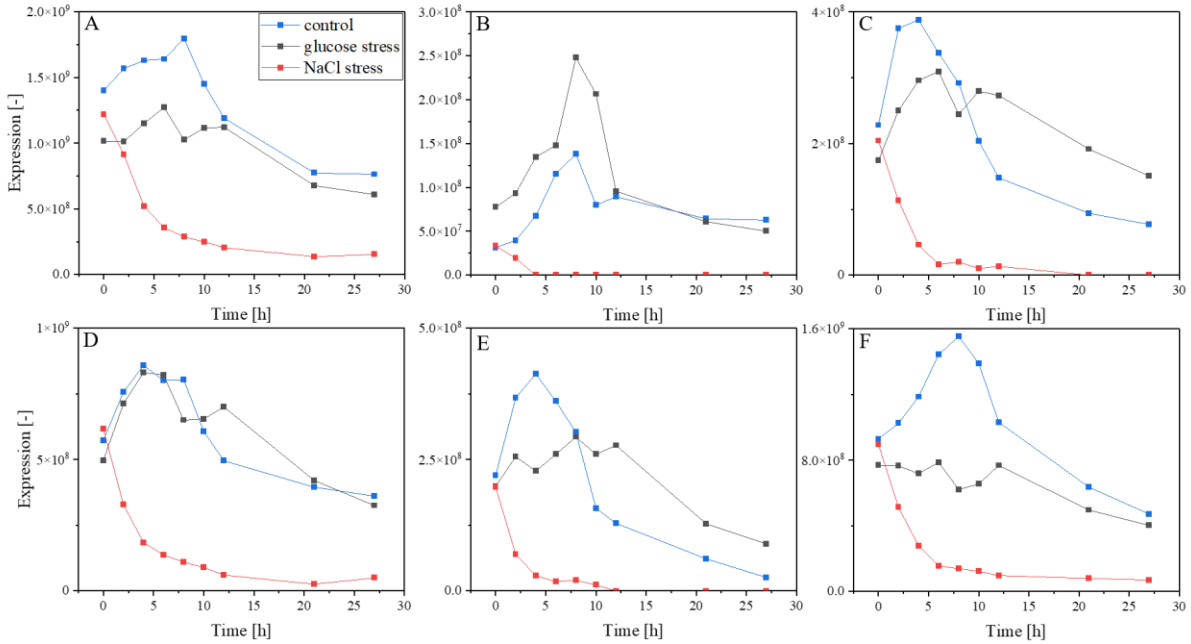


Figure 14: Relative expression of six exemplary proteins from a single operon associated with cell motility in *B. favorum* TMW 2.1880 under glucose or NaCl stress (PGAP annotation in parenthesis): (A) CPA57_06095 (chemotaxis protein CheW), (B) CPA57_06190 (flagellar hook-basal body complex protein FliE), (C) CPA57_06305 (flagellar biosynthesis protein FlgA), (D) CPA57_06355 (flagellar motor switch protein FliG), (E) CPA57_06385 (flagella basal body P-ring formation protein FlgA), (F) CPA57_06435 (flagellar basal body-associated protein FliL).

The results of the proteomic experiment in *B. favorum* TMW 2.1880 can be summarized as follows: several transporter proteins were identified that were differentially expressed under glucose or NaCl stress. Negative and positive regulations of these proteins were detected for both types of stress. Five proteins were identified that were up-regulated under glucose stress: three enzymes with oxidoreductase activity, a key enzyme of proline metabolism, and a molecular chaperone. NaCl stress induced the expression of a T4SS. In addition, several proteins of a single operon associated with cell motility were downregulated.

3.6.4 Analysis of intercellular metabolites

To investigate another aspect of the cellular response of *B. favorum* TMW 2.1880 to glucose and NaCl stress, an experiment was performed to analyze intracellular metabolites (Section 2.7). For this purpose, an over-night culture was used to inoculate 10 mL of glucose stress medium and NaCl stress medium at an OD between 0.1 and 0.2. A culture in fresh LMG 404 medium was used as a reference. After 5 h the cells were harvested, washed and prepared for the metabolomics analysis. The LC/MS analysis, data processing and quality check were performed externally. Some of the

results are visualized in Figure 15. All other intracellular metabolites that were detected with significantly different quantities are shown in the Supplementary Table S4 and S5.

Several carbohydrates were detected in significantly different quantities. Molecules with the molecular formula $C_5H_{12}O_5$ were significantly more abundant in *B. favorum* TMW 2.1880 cells under glucose stress than under NaCl stress or in the reference medium. This molecular formula corresponds to the sugar alcohols arabitol, xylitol or ribitol. Other sugar alcohols with the molecular formula $C_4H_{10}O_4$ (erythritol or threitol) are also significantly more present under glucose stress than under NaCl stress or in the reference medium, with the difference that they are also significantly increased under NaCl stress compared to the reference medium. The same distribution (control < NaCl stress < glucose stress) was also detected for carbohydrates with the molecular formulas $C_6H_{12}O_6$ and $C_5H_{10}O_5$ and for the amino acid proline.

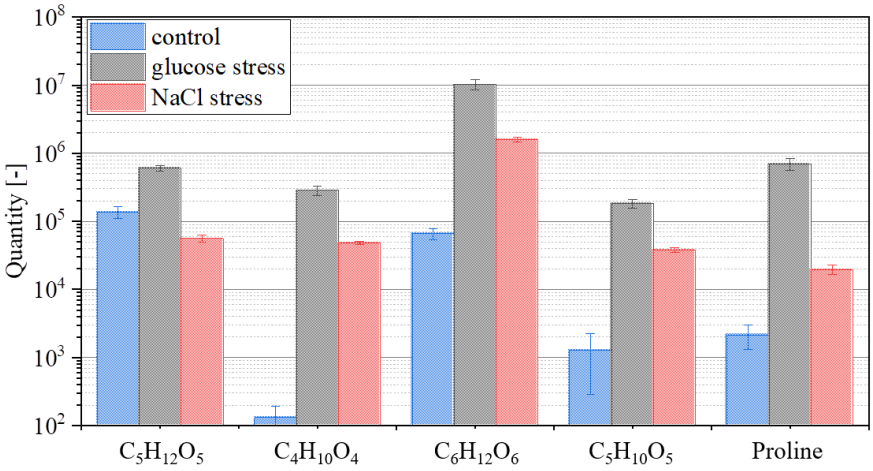


Figure 15: Relative quantities of different metabolites in *B. favorum* TMW 2.1880 cells under glucose stress (400 g/L), NaCl stress (10 g/L) or in the reference medium.

3.7 *Bombella* spp. as starter cultures in the fruit juice industry

Since strains of the genus *Bombella* have a high tolerance to sugar and are related to other acetic acid bacteria used in the food industry, an application as starter culture strains in fruit juice fermentations was considered. The general idea was to ferment fruit juice concentrates to obtain new flavor profiles and lower the sugar content.

Fruit juices have other microbial hurdles besides high sugar levels, such as low pH and the presence of secondary metabolites such as polyphenols. To test whether *Bombella* spp. can persist in these matrices, growth experiments with eight different *Bombella* strains were done in four different

commercial fruit juices: grape juice, apple juice, sour cherry nectar and orange juice from concentrate (Figure 16). 10 mL of each juice was inoculated with overnight cultures gown in LMG 404 medium and incubated at 30 °C and 200 rpm. The cell count was determined after 0, 7 and 24 h. An increase of the cell count was observed for each strain in each juice. For some strains and juices, growth was observed until 7 h, but at 24 h a decrease of the cell counts was measured. The highest cell counts were reached in orange juice with over 10^9 CFU/mL. Overall, it can be concluded that *Bombella* spp. may be generally suitable for the fermentation of various fruit juices, since growth was measured in all cases.

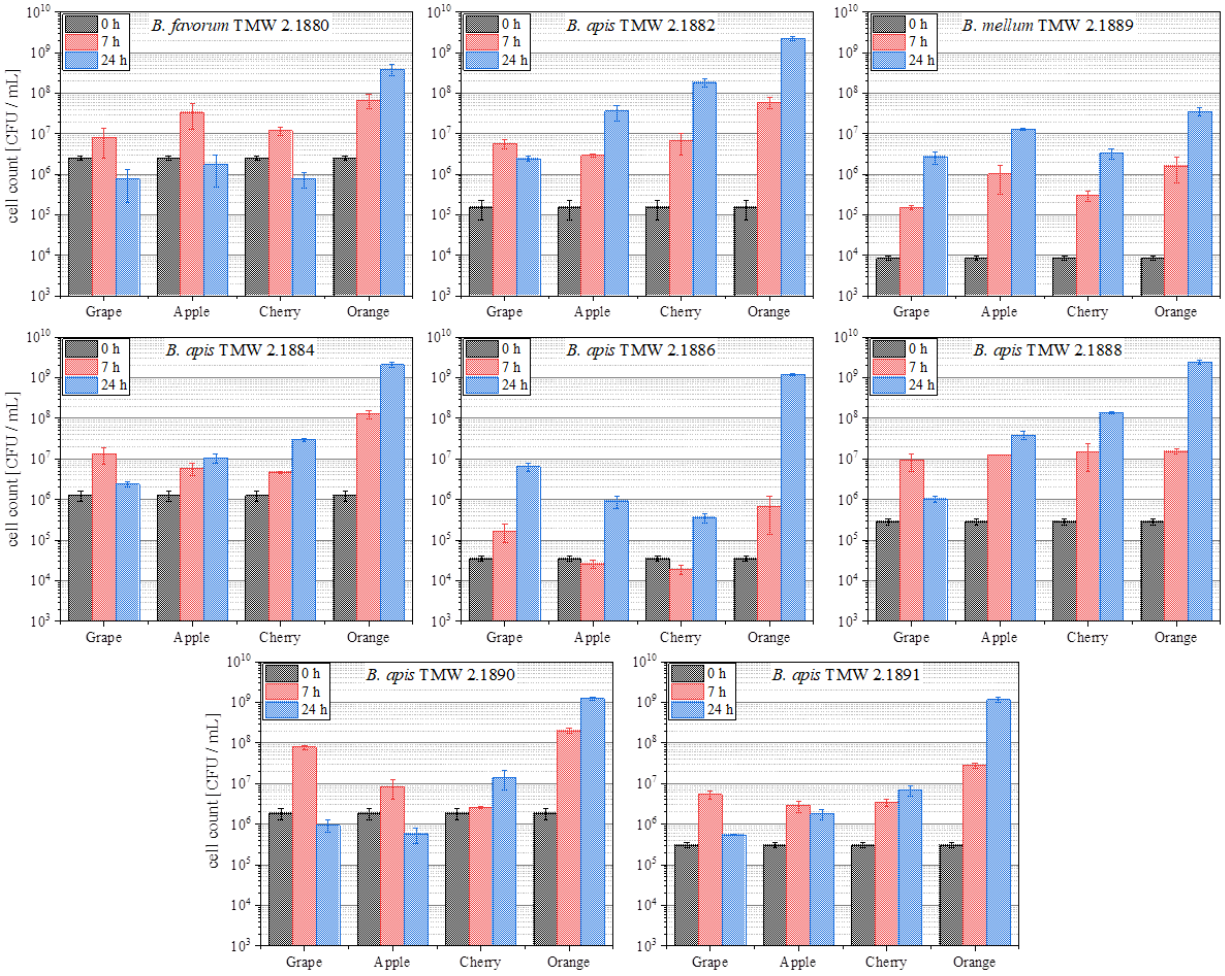


Figure 16: Growth behavior of *Bombella* strains in grape juice, apple juice, sour cherry nectar and orange juice from concentrate. Cultures were incubated at 30 °C and 200 rpm.

3.7.1 Adaptability of *B. favorum* TMW 2.1880 through UV mutagenesis

It is common practice to use random mutagenesis tools to adapt starter culture strains to the matrix to be fermented. To test whether the growth of *B. favorum* TMW 2.1880 could be improved in high concentrated fruit juices, the strain was adapted to high glucose concentrations (400 g/L) through UV mutagenesis (Section 2.1.6).

Growth experiments in glucose concentrations from 100 to 500 g/L were performed with the adapted strain and the wild-type strain (Figure 17). Until 400 g/L glucose, the adapted strain showed a shorter lag-phase and reached higher maximum OD values. In the medium containing 500 g/L glucose, the adapted strain reached the exponential growth phase faster, but no increased maximum OD was measured anymore. It appears that a general adaptation of the strain to the growth conditions of a shake flask culture occurred, but no adaptation to higher glucose concentrations.

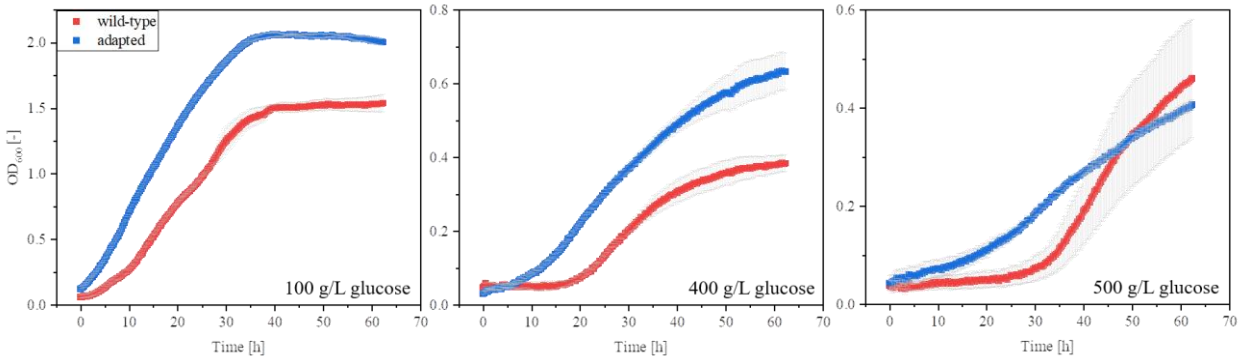


Figure 17: Growth of the wild-type strain and the adapted strain of *B. favorum* TMW 2.1880 in medium with 100, 400 and 500 g/L glucose. The 250 µL cultures were incubated at 30 °C.

Growth experiments in grape juice concentrate were performed to test whether the adaption also led to better growth in fruit juice matrices. For this purpose, grape juice concentrate was diluted with distilled water to total sugar concentrations between 100 and 500 g/L and used as culture media (Figure 18). The adapted strain showed a shorter lag-phase and reached higher maximum OD values in the grape concentrates between 100 and 300 g/L total sugars. At 400 g/L total sugars no growth was observed for the wild-type strain, but for the adapted strain. Thus, adaptation by UV mutagenesis to media containing 400 g/L glucose resulted not only in improved growth in grape juice concentrates, but also in an increase in the maximum concentration at which growth could still occur. No growth was observed at 500 g/L total sugars.

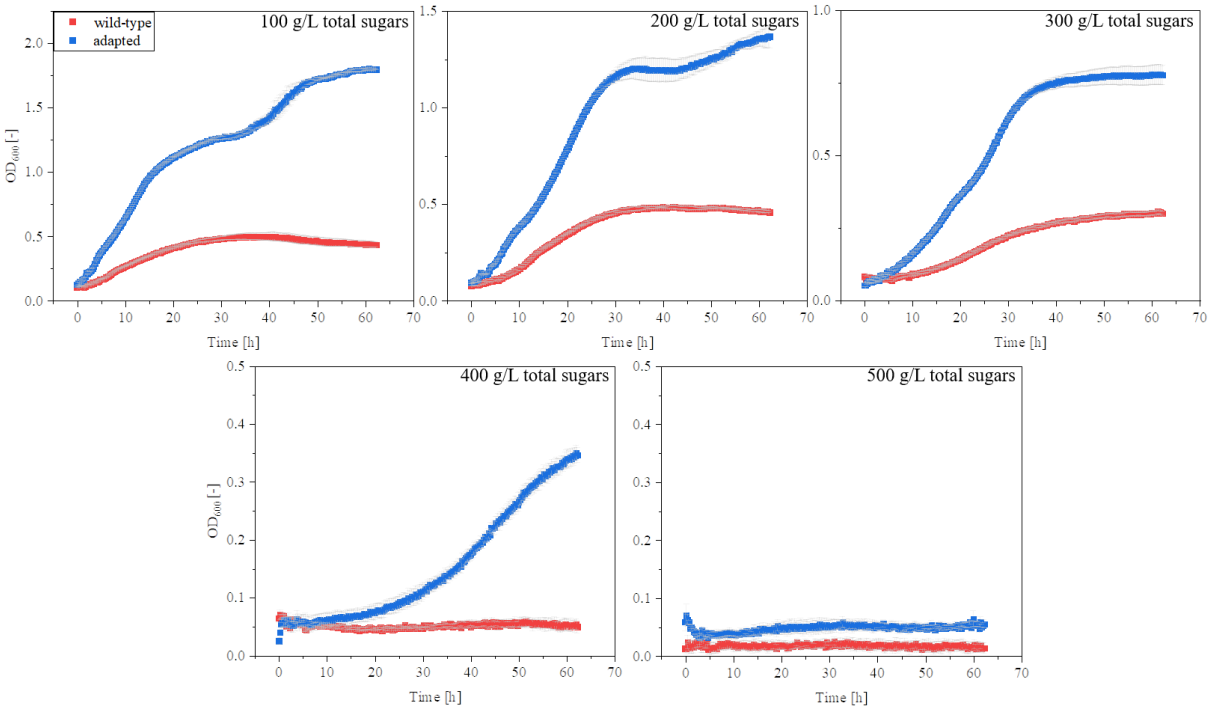


Figure 18: Growth of the wild-type strain and the adapted strain of *B. favorum* TMW 2.1880 in grape juice concentrate with total sugar concentrations of 100, 200, 300, 400 and 500 g/L. The 250 µL cultures were incubated at 30 °C.

3.7.2 Differentially expressed proteins of the adapted strain

A proteomics experiment (Section 2.6) was performed to investigate the cellular changes that led to the altered growth characteristics of the adapted *B. favorum* TMW 2.1880 strain. Since higher culture volumes are needed for proteomics experiments, it was first tested how the strains behaved after a scale-up to 100 mL cultures. Growth in medium with 400 g/L glucose and in LMG 404 medium was tested. The results are shown in Figure 19. In both media, the adapted strain showed an earlier transition to the exponential growth phase compared to the wild-type strain. While in smaller volumes the adapted strain also achieved higher OD values, this effect was no longer detectable in 100 mL cultures. It can be concluded that an adaption effect was also measurable at higher culture volumes, but this effect is considerably weaker than in the experiments at microplate scale.

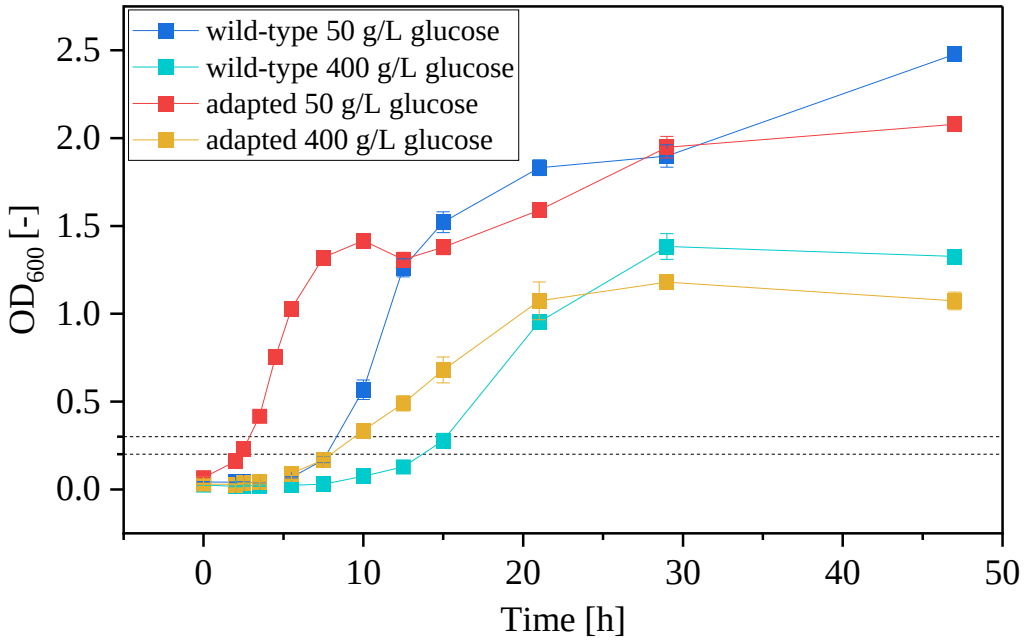


Figure 19: Growth of the wild-type strain and the adapted strain of *B. favorum* TMW 2.1880 in media with 400 g/L glucose and 50 g/L glucose. The 100 mL cultures were incubated at 30 °C and shaken at 100 rpm. The two dashed lines mark the OD values 0.2 and 0.3.

For the proteomics experiment (Section 2.6), medium with 400 g/L glucose was inoculated with OD values of 0.05. The cultures were harvested at OD values between 0.2 and 0.3 (see Figure 19, dashed lines). At these OD values, the culture should be at the beginning of the exponential growth phase, i.e. the cells should have adapted optimally to the medium. The LC/MS measurements, data processing and quality check of the proteomics data were performed externally. In total, 1,504 proteins were detectable out of 1,707 theoretically possible coding sequences.

Compared to the wild-type strain of *B. favorum* TMW 2.1880 the adapted strain expressed eight proteins significantly higher and 66 proteins significantly lower during growth in 400 g/L glucose (Figure 20).

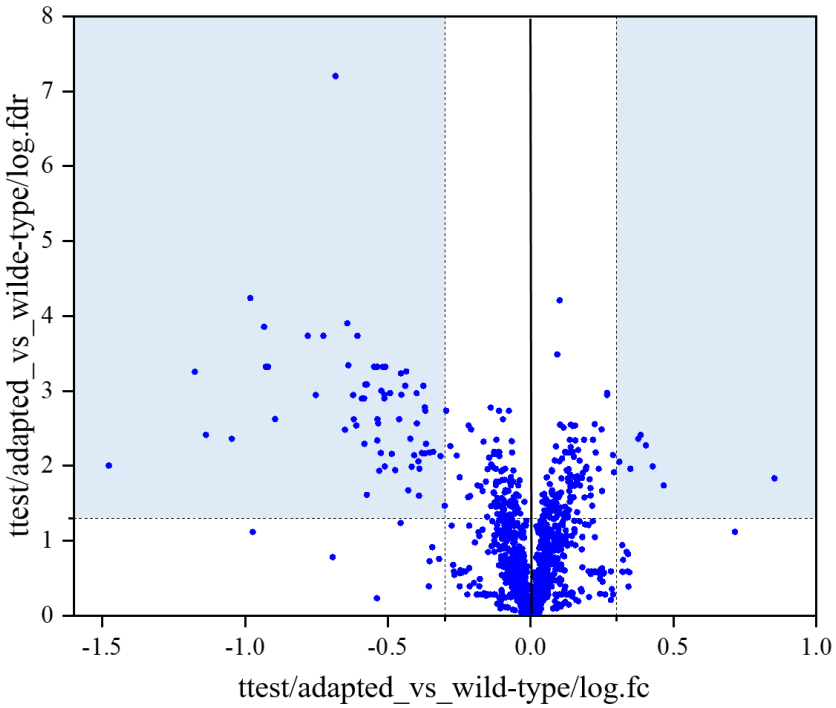


Figure 20: Volcano plot of differentially expressed proteins between the adapted strain and the wild-type strain of *B. favorum* TMW 2.1880, when grown in glucose stress medium. Proteins within the light blue background are significantly differentially expressed. log.fdr = log10 false discovery rate; log.fc = log10 fold change.

All significantly differentially expressed proteins were submitted to the EggNOG-Mapper webserver (Section 2.3.3) to obtain functional annotations based on the database of Clusters of Orthologous Genes (COGs, Figure 21). This was done to determine which cellular functions were affected by the UV mutagenesis. A table with the respective locus tags, COGs, and statistical data is shown in Supplementary Table S6. Of the 8 proteins that were significantly higher expressed, 6 could be assigned to functional groups. Of the 66 proteins that were significantly lower expressed, 46 could be assigned to a functional group. The proteins were classified into 11 different functional groups in total. The most relevant groups were "Signal transduction mechanisms" with nine down-regulated proteins, "Cell wall/membrane/envelope biogenesis" with five down-regulated proteins and one up-regulated protein and "Cell motility" with 26 down-regulated proteins.

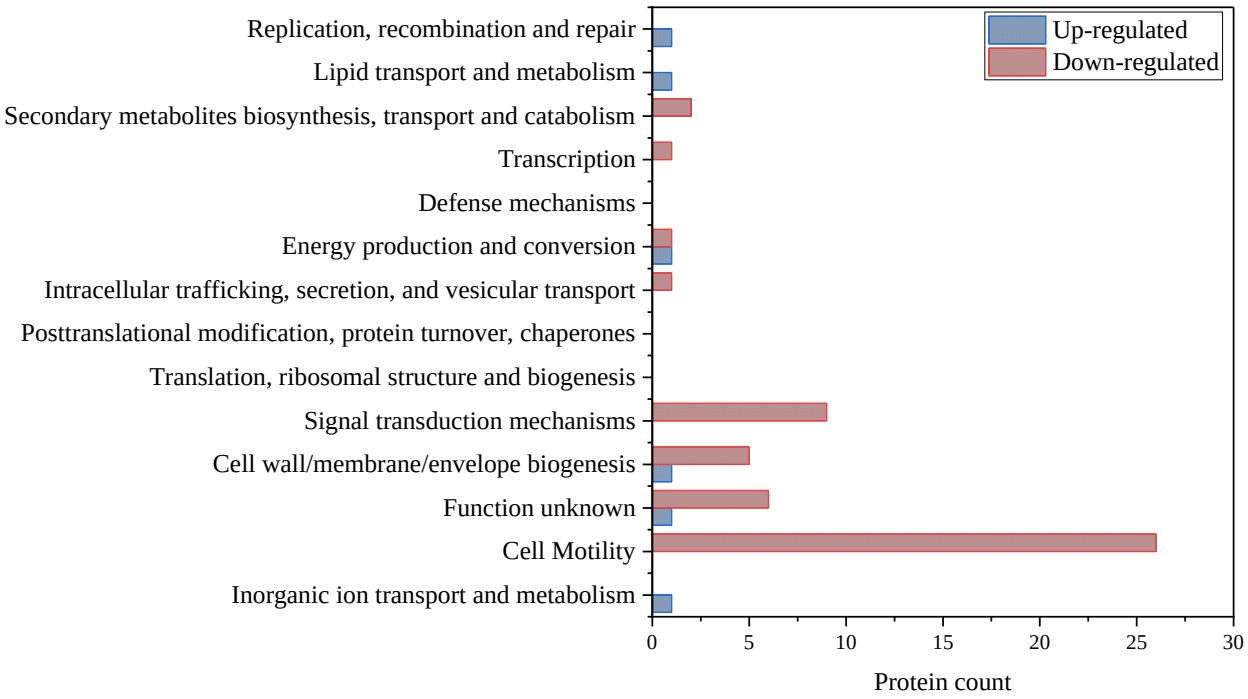


Figure 21: Classification of significantly differentially expressed proteins into functional groups using the database of COGs. Comparison of the adapted strain to the wild-type strain of *B. favorum* TMW 2.1880 when grown in glucose stress medium (400 g/L).

51 of the 66 lower expressed proteins by the adapted strain are located on the operon associated with flagella regulation and assembly that was already mentioned in section 3.6.3. It is assumed that down-regulation of cell motility with its complex protein structures could lead to energy savings that could be used for other cellular processes. This could explain the changes in the growth behavior of the adapted strain. Only 10 proteins not located on the operon could be assigned to functional groups. However, none of them could be associated with the altered growth behavior.

To investigate whether similar cellular mechanisms led to the improved growth of the adapted strain in grape juice concentrates, the same proteomics experiment setup was used with grape juice concentrate with a total sugar content of 200 g/L instead of the glucose stress medium. Compared to the wild-type strain of *B. favorum* TMW 2.1880 the adapted strain expressed 18 proteins significantly higher and 64 proteins significantly lower during growth in grape juice concentrate (Figure 22). A table with the respective locus tags, COGs, and statistical data is shown in Supplementary Table S7.

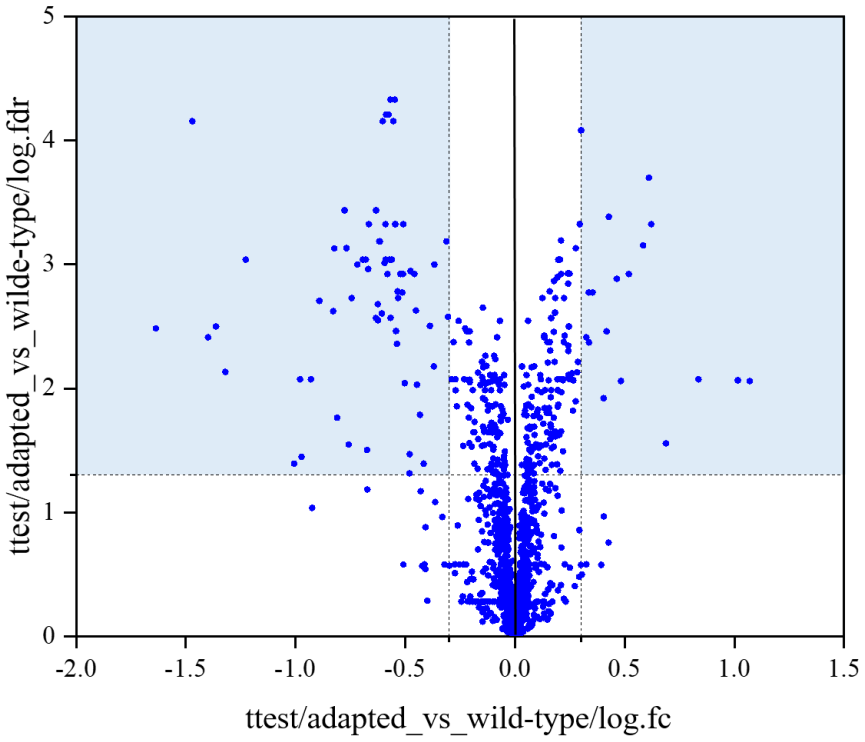


Figure 22: Volcano plot of differentially expressed proteins between the adapted strain and the wild-type strain of *B. favorum* TMW 2.1880, when grown in grape juice concentrate with a total sugar content of 200 g/L. Proteins within the light blue background are significantly differentially expressed. log.fdr = log10 false discovery rate; log.fc = log10 fold change.

Of the 18 proteins that were significantly higher expressed, 14 could be assigned to functional groups. Of the 64 proteins that were significantly lower expressed, 54 could be assigned to functional groups. In total, the proteins were classified into 12 different functional groups (Figure 23). As with growth in glucose stress medium, the functional groups with the most differentially expressed proteins were "Signal transduction mechanisms" with eleven down-regulated proteins and one up-regulated protein, "Cell wall/membrane/envelope biogenesis" with seven down-regulated proteins and one up-regulated protein and "Cell motility" with 28 up-regulated proteins. Again, the majority of the proteins that are significantly down-regulated in the adapted strain are located on the operon associated with flagella (72 %).

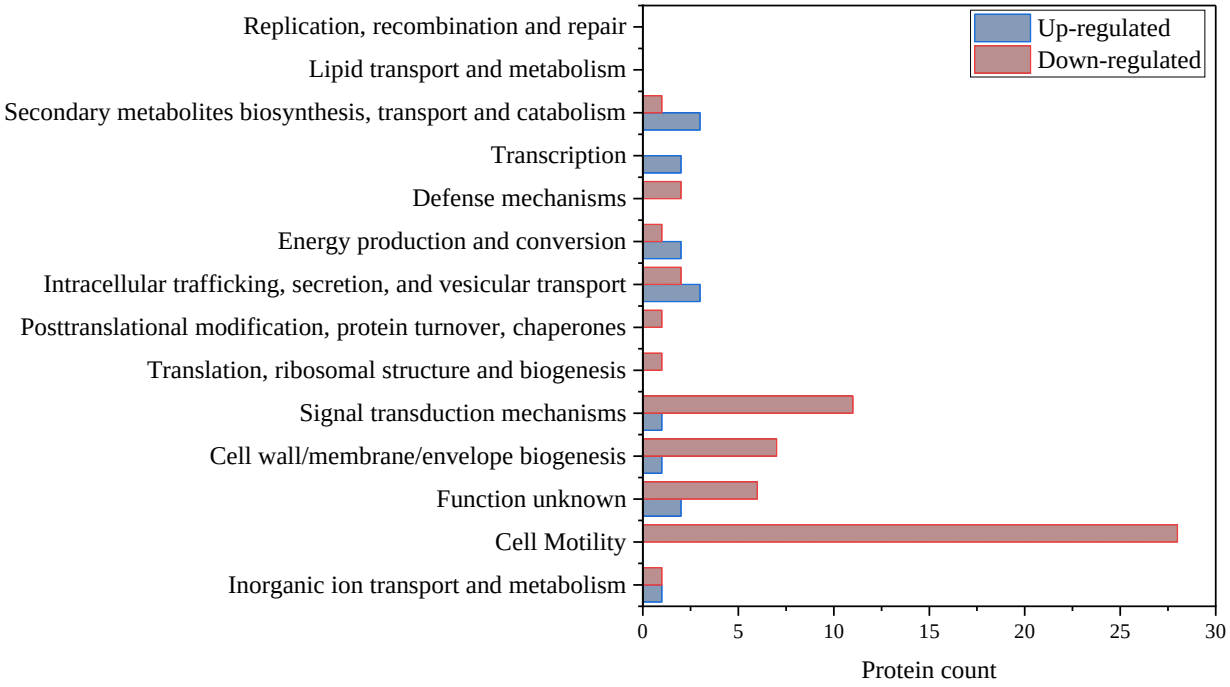


Figure 23: Classification of significantly differentially expressed proteins into functional groups using the database of COGs. Comparison of the adapted strain to the wild-type strain of *B. favorum* TMW 2.1880 when grown in grape juice concentrate with a total sugar content of 200 g/L.

Of the 64 proteins that are significantly less expressed by the adapted strain when growing in grape juice concentrate, 53 (83 %) were also less expressed in the glucose stress medium. Only one of the upregulated proteins was also identified under glucose stress. However, the total overlap of differentially expressed proteins is at 65 %.

It can therefore be concluded that adaptation to a single stressor, in this case high glucose concentrations, also leads to improved growth in grape juice concentrate. However, the major influenced cellular mechanisms are not, as expected, related to the actual adaptation to high sugar concentrations, but appear to be due to a more general adaption to the shaking flask cultivation. This is mainly achieved by the reduced expression of proteins associated with the regulation, assembly and operation of flagella.

3.8 Formation of extracellular polysaccharides by *Bombella* spp.

Note: Parts of this section have already been published in Härer et al. (2023a).

In addition to their use as starter culture strains in the food industry, acetic acid bacteria have biotechnological applications because of their ability to produce EPS. The formation of such substances is typically tested by plating the strains on agar plates containing the respective sugar which can serve as substrate for the enzymatic reaction. Slime formation around the colonies indicates the formation of EPS.

To test whether strains of the genus *Bombella* can form EPS from sucrose, 8 at the time available strains were spread on agar plates containing 50 g/L sucrose and 10 g/L yeast extract and incubated for 4 days at 30 °C. Two strains were forming slime: *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889. No slime was formed when the strains were grown on a mixture of 25 g/L glucose and 25 g/L fructose (monosaccharides of sucrose) or on 50 g/L raffinose. In other literature, EPS of acetic acid bacteria are isolated from liquid cultures (Jakob et al. 2013), since this was not possible with the *Bombella* strains, the EPS were recovered directly from the plates as described in section 2.5.1.

3.8.1 Monosaccharide composition and glycosidic linkages

The monosaccharide composition and glycosidic linkages of the recovered EPS were analyzed externally. Analysis of the monosaccharide composition (Section 2.5.2) revealed that the main component was fructose, i.e. the EPS are fructans. Galactose and glucose were detected only in traces below 2 %. Using NMR-spectroscopy (Section 2.5.3), levan-derived signals were detected for both fructans. In addition, determination of glycosidic linkages by methylation analysis (Section 2.5.4) provided further insight into the portions of linkages (Table 11) and revealed that both fructans are not solely 2,6-linked but also contain about 10 % of 1,2-linked Fru*f* units. Furthermore, both fructans contain 7 % of branched units (1,2,6-Fru*f*).

Table 11: Glycosidic linkages [mol-%] of fructans determined by methylation analysis.

Glycosidic linkages	*B. apis* TMW 2.1884	*B. mellum* TMW 2.1889
2,6-Fru*f*	72 mol-%	54 mol-%
1,2-Fru*f*	8 mol-%	15 mol-%
1,2,6-Fru*f*	7 mol-%	7 mol-%
t-Fru*f*	13 mol-%	24 mol-%

3.8.2 Molecular weight determination

AF4-MALLS (Section 2.5.5) was used to determine the molecular weight of the recovered fructans from *Bombella*. The average molecular weight of the *B. apis* TMW 2.1184 and *B. mellum* TMW 2.1189 fructan was 77.6 mDa (± 0.2 %) and 80.5 mDa (± 0.5 %), respectively. Thus, the fructans are high molecular weight (HMW) polysaccharides. The molecular weight distribution is shown in Figure 24 as the differential and cumulative weight fraction.

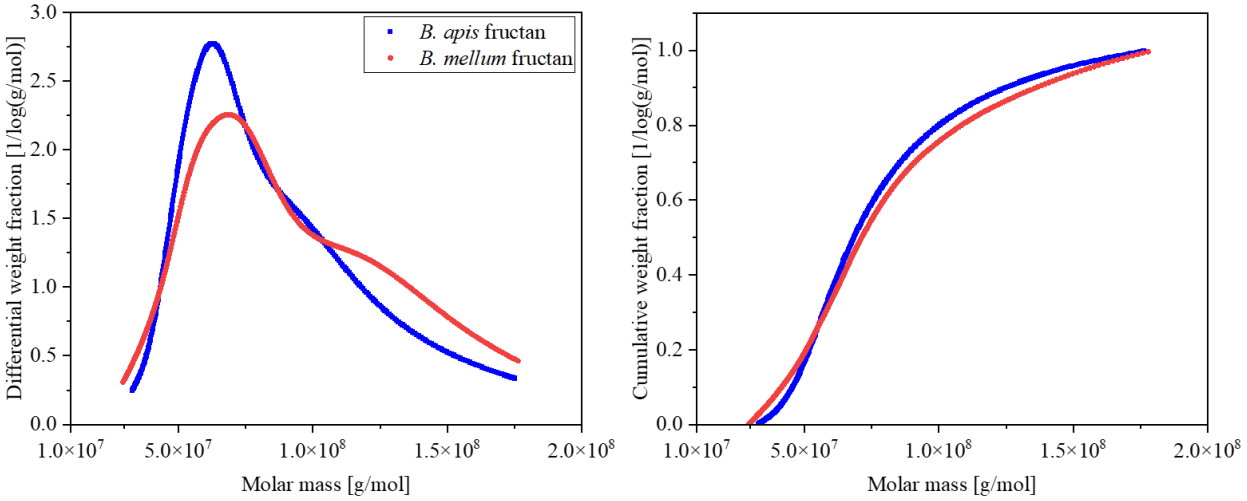


Figure 24: Differential (left) and cumulative (right) molecular weight distribution of native *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889 fructans recovered from sucrose-containing agar plates determined with AF4-MALLS.

3.8.3 Identification of the *Bombella* fructosyltransferase

In order to identify the enzyme that catalyzes fructan synthesis, the *Bombella* genomes were analyzed *in silico* for corresponding genes. For this purpose, the genomes of all eight strains were annotated with the DBcan webserver (Section 2.3.3), a tool that identifies all carbohydrate active enzymes in the respective genomes. Typically, levan synthesis of acetic acid bacteria is catalyzed

by enzymes of the GH 68 family. Since no enzymes of the GH 68 family were identified, enzymes from the GH 32 family were also included in the search. A GH 32 family enzyme was identified in all genomes. However, for *B. apis* TMW 2.1884, *B. apis* TMW 2.1891 and *B. mellum* TMW 2.1889 additional GH 32 family enzymes were identified with the respective locus tags DTI93_RS00530 (445 amino acids (AAs)), DTJ15_RS01635 (476 AAs) and CPA56_RS00775 (520 AAs).

The percentage identity between the three enzymes is between 87.3 and 94.7 % and below 23.7 % towards the other GH 32 family enzymes (Table 12). In the *B. apis* TMW 2.1891 open reading frame (ORF) DTI93_RS00530, one nucleotide is missing after position C1376, which probably leads to the expression of a non-functioning protein due to a frameshift. This mutation explains why the respective strain did not form EPS. The affected site in the *B. apis* TMW 2.1891 genome was sequenced by Eurofins Genomics (Ebersberg, Germany) for confirmation.

Table 12: Percentage identity [%] of *Bombella* GH 32 family enzymes calculated with Clustal Omega. Green color indicates percentage identities above 87 %. Yellow color indicates percentage identities below 24 %. 1 – 8: GH 32 family enzymes present in all *Bombella* genomes. 1 = *B. favorum* TMW 2.1880 (CPA57_RS07755), 2 = *B. mellum* TMW 2.1889 (CPA56_RS07355), 3 = *B. apis* TMW 2.1891 (DTJ15_RS07680). 4 = *B. apis* TMW 2.1888 (DTQ13_04050), 5 = *B. apis* TMW 2.1884 (DTI93_RS04755), 6 = *B. apis* TMW 2.1882 (CO583_RS08065), 7 = *B. apis* TMW 2.1890 (CPA54_RS07155), 8 = *B. apis* TMW 2.1886 (DTJ06_07900). 9 – 11: Additional GH 32 family enzymes. 9 = *B. mellum* TMW 2.1889 (CPA56_RS00775), 10 = *B. apis* TMW 2.1884 (DTI93_ RS00530), 11 = *B. apis* TMW 2.1891 (DTJ15_RS01635). NCBI accession number are given in parentheses.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	100	91.37	90.98	90.82	90.82	90.98	90.98	90.98	23.67	22.03	22.59
2	91.37	100	92.97	92.97	92.97	93.13	93.13	93.13	22.81	21.68	21.88
3	90.98	92.97	100	99.53	99.68	99.68	99.68	99.84	22.6	21.44	21.88
4	90.82	92.97	99.53	100	99.53	99.84	99.84	99.68	22.81	21.64	22.12
5	90.82	92.97	99.68	99.53	100	99.68	99.68	99.84	22.81	21.64	22.12
6	90.98	93.13	99.68	99.84	99.68	100	100	99.84	22.81	21.64	22.12
7	90.98	93.13	99.68	99.84	99.68	100	100	99.84	22.81	21.64	22.12
8	90.98	93.13	99.84	99.68	99.84	99.84	99.84	100	22.81	21.64	22.12
9	23.67	22.81	22.6	22.81	22.81	22.81	22.81	22.81	100	90.56	87.34
10	22.03	21.68	21.44	21.64	21.64	21.64	21.64	21.64	90.56	100	94.74
11	22.59	21.88	21.88	22.12	22.12	22.12	22.12	22.12	87.34	94.74	100

To demonstrate that the synthesis of *Bombella* spp. fructans is catalyzed by the identified additional GH 32 family enzymes, the *B. mellum* TMW 2.1889 ORF CPA56_RS00775 as well as two versions

of the *B. apis* TMW 2.1884 ORF DTI93_RS00530 were cloned (Section 2.2.2) and expressed in *E. coli* Top10. The first version corresponds to the original ORF, the second version is extended by 357 bases (119 AAs) because, according to the whole genome data, another ORF is also conceivable (Supplementary Data S8). An *E. coli* Top10 strain transformed with an unmodified expression plasmid was always included as a control. Purification of a useable amount of the heterologously expressed proteins by affinity chromatography failed due to poor expression and the strong tendency to form inclusion bodies. However, slime formation was observed for the *E. coli* strain expressing the extended version of the *B. apis* TMW 2.1884 protein when grown on agar plates containing the substrate sucrose, the inducer arabinose and sorbitol to facilitate correct protein folding (Section 2.5.1). Formed fructans were recovered from the agar plates and subjected to methylation analysis (Section 2.5.4). The analysis revealed that comparable portions of all three linkage types (2,6-Fru*f*, 1,2-Fru*f* and 1,2,6-Fru*f*) occur in the fructan natively produced by *B. apis* TMW 2.1884 and in the fructan produced by the *E. coli* strain expressing the extended version of the GH 32 family enzyme (Table 13). With a high level of confidence, it is postulated that the heterologous expressed enzyme is the FTase that catalyzes fructan formation in *B. apis* TMW 2.1884. Accordingly, it is assumed that in *B. mellum* TMW 2.1889 the ORF CPA56_RS00775 encodes for the corresponding FTase.

Table 13: Glycosidic linkages [mol-%] of native *B. apis* TMW 2.1884 fructan and fructan produced by *E. coli* Top10 strain expressing an extended version of the *B. apis* TMW 2.1884 GH 32 family enzyme determined by methylation analysis.

Glycosidic linkages	Native	*E. coli*
2,6-Fru*f*	72 mol-%	65 mol-%
1,2-Fru*f*	8 mol-%	11 mol-%
1,2,6-Fru*f*	7 mol-%	10 mol-%
t-Fru*f*	13 mol-%	14 mol-%

3.8.4 Sequence analysis of the *Bombella* fructosyltransferase

For a phylogenetic analysis (Section 2.3.4) of the *Bombella* FTases, several GH 32 and GH 68 family enzyme sequences were obtained from the NCBI database. Actual enzymatic activity of the enzymes used was demonstrated in corresponding publications. The GH 68 family was represented by levansucrases from *Gluconobacter albidus* (Jakob et al. 2020), *Kozakia baliensis* (Brandt et al. 2016), *Gluconacetobacter diazotrophicus* (Martínez-Fleites et al. 2005), *Halomonas smyrnensis*

(Poli et al. 2009), *Pseudomonas syringae* (Visnapuu et al. 2011), *Bacillus subtilis* (Porras-Domínguez et al. 2015), *Leuconostoc mesenteroides* (Kang et al. 2005) and *Limosilactobacillus reuteri* (Ni et al. 2018). Accordingly, enzymes from *Bacillus licheniformis* (Porras-Domínguez et al. 2014), *Bacillus subtilis* (Jensen et al. 2016), *Gluconacetobacter diazotrophicus* (Menéndez et al. 2004), *Microbacterium* sp. AL-210 (Cha et al. 2001), *Paenarthrobacter ureafaciens* (Song et al. 2000), *Microbacterium laevaniformans* (Song et al. 2002) and *Streptomyces exfoliatus* (Yokota et al. 1993) were used for the levan-degrading GH 32 family. The corresponding phylogenetic tree with all NCBI accession numbers is shown in Figure 25. The tree shows a clear grouping of the two GH enzyme families, independent of the taxonomic relationship of the host organisms. The uniqueness of *Bombella* FTases is shown by the formation of its own branch in the phylogenetic tree, which can be assigned to the GH 32 family.

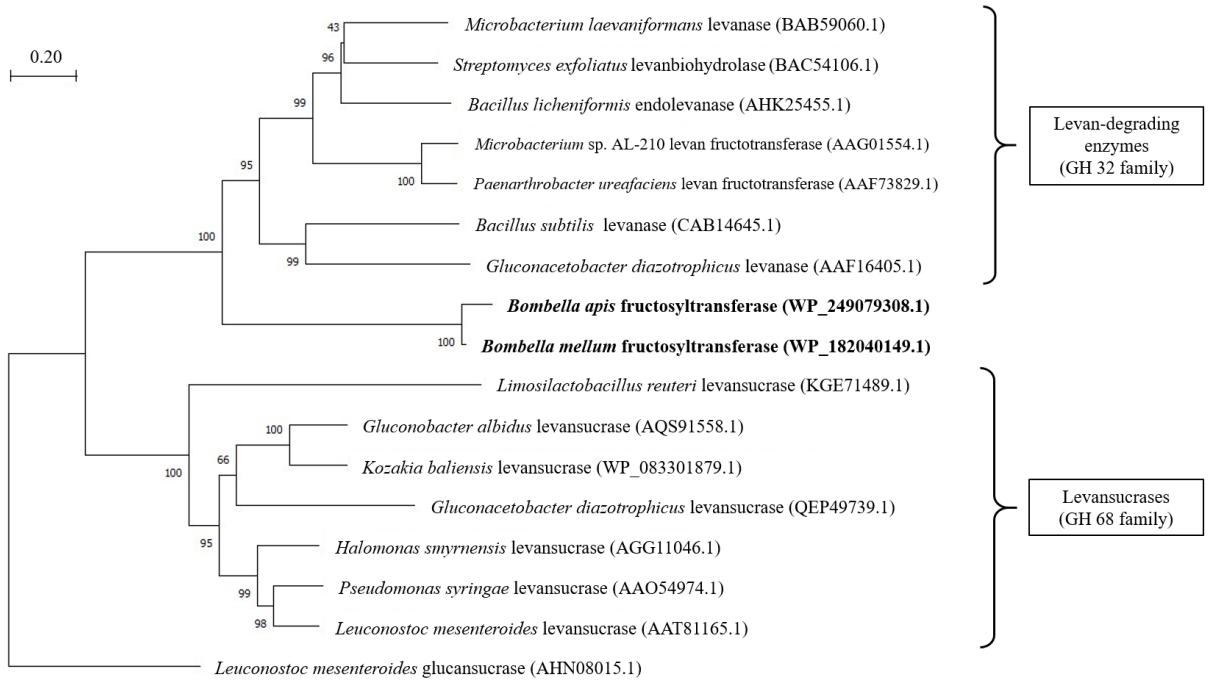


Figure 25: Phylogenetic tree of bacterial GH 32 and GH 68 family enzymes with *Bombella* fructosyltransferases using the Neighbor-Joining method (Saitou and Nei 1987) in MEGA X (Kumar et al. 2018). Scale represents the number of amino acid substitutions per site. Numbers at nodes indicate bootstrap percentages using 1000 replicates. *Granulibacter bethesdensis* CGDNIH1^{T} was used as the outgroup. NCBI accession numbers are given in parentheses.

Crystal structure models of the *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889 FTases (Figure 26) were constructed with the SWISS-MODEL webserver (Template SMTL ID: 3rwk.1, section

2.3.5). Additionally, visualized crystal structures of a levansucrase (GH 68 family) from *Gluconacetobacter diazotrophicus* (SMTL ID: 1w18.1) and a levan fructotransferase (GH 32 family) from *Paenarthrobacter ureafaciens* (SMTL ID: 4fff.1) are shown for comparison. Protein domains predicted by InterPro were added manually (Section 2.3.5). Five-bladed *β*-propeller catalytic domains (yellow) were detected in all enzymes. The C-terminal domains of the three GH 32 enzymes form *β*-sandwich modules (turquoise) which clearly distinguishes them from the GH 68 family enzyme. Catalytic triads are composed of Asp75, Asp216 and Glu263 for *B. apis*, Asp31, Asp172 and Glu219 for *B. mellum*, Asp73, Asp247 and Glu339 for *Ga. diazotrophicus* and Asp14, Asp146 and Glu196 for *P. ureafaciens.*

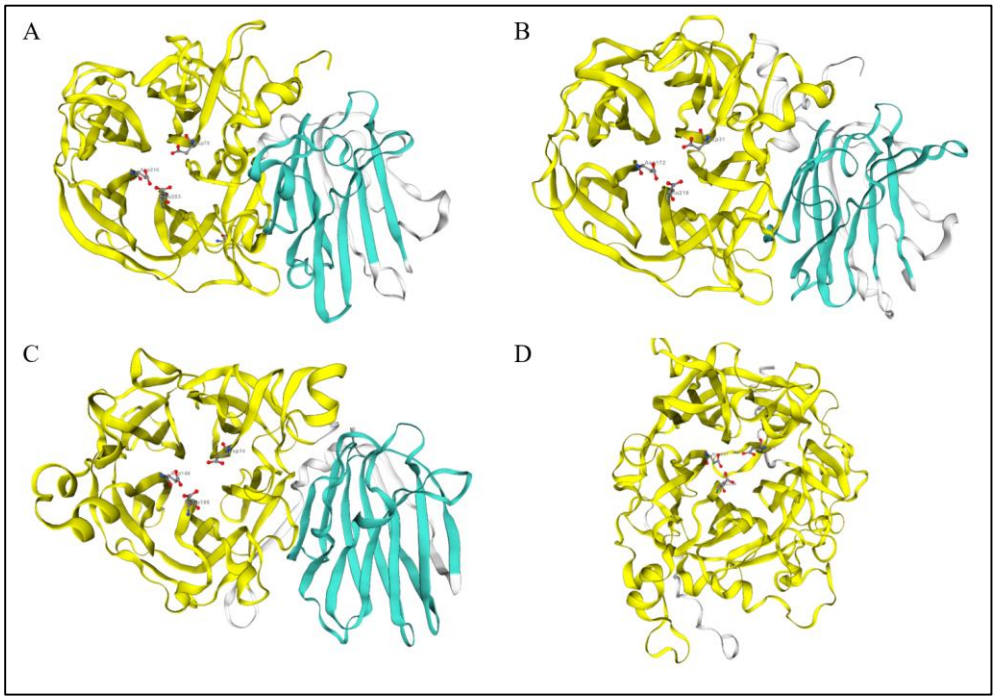


Figure 26: Protein crystal structure models of (A) *B. apis* TMW 2.1884 and (B) *B. mellum* TMW 2.1889 fructosyltransferases. Models were constructed with the SWISS-MODEL (Template SMTL ID: 3rwk.1). The GH 32 *C*-terminal domains are colored turquoise (*B. apis*: 444 – 551; *B. mellum*: 400 – 507) and *N*-terminal five-bladed *β*-propeller catalytic domains yellow (*B. apis*: 50 – 393; *B. mellum*: 24 – 351). The highlighted predicted catalytic triad consists of Asp75, Asp216, and Glu263 for *B. apis* and Asp31, Asp172, and Glu219 for *B. mellum*. For comparison, visualized crystal structures models of a (C) levan fructotransferase (GH 32 family) from *Paenarthrobacter ureafaciens* (SMTL ID: 4fff.1) and a (D) levansucrase (GH 68 family) from *Gluconacetobacter diazotrophicus* (SMTL ID: 1w18.1) are shown.

Both enzyme sequences of *Bombella* FTases were screened for conserved motifs of other microbial FTases (Velázquez-Hernández et al. 2009). A total of eleven motifs with different functions were analyzed. The results are shown in Table 14. All motifs were found in both enzyme sequences, but

the corresponding regions all show differences from the consensus sequences described in literature. All identified FTase motifs are located on the *N*-terminal five-bladed *β*-propeller catalytic domain.

Table 14: Conserved motifs among microbial fructosyltransferases. Modified from (Velázquez-Hernández et al. 2009). Bold characters in the *Bombella* sequence indicate consensus positions, underlined characters indicate amino acids with properties similar to the consensus sequence. Functions were obtained from (Meng and Fütterer 2003; Martínez-Fleites et al. 2005; van Hijum et al. 2006). *Motif designation and amino acids positions are relative to *Bacillus subtilis* levansucrase sequence (NCBI accession number: P05655).

Motif number	Motif designation	Consensus sequence	Position*	*Bombella* sequence	Position *Bombella* (*apis* / *mellum*)	Function
I	VWDSW	(V/L)WD(T/S)(W/M)	84–88	**LW**R**TW**	(87–91 / 43–47)	Catalytic nucleophile, conserved in GH 68 family
II	EWSGS	(E/Q)W(A/S)G(T/S)	162–166	Y**WSGS**	(145-149 / 101–105)	Sucrose box I, conserved in GH 68 family
III	DG	DG	172–173	R**G**	(160-161 / 116–117)	Involved in acceptor recognition
IV	LFYTD	L(F/Y)YT(D/C)	177–181	V**YY**YI	(165-169 / 121–125)	Sucrose box II and acceptor recognition
V	RDP	RDP	246–248	**RD**C	(215-217 / 171–173)	Catalytic centre, stabilizes transition state and contributes to sucrose hydrolysis and fructan polymerization
VI	YLVFE	Y(M/L)VFE	258–262	FA**F**FR	(238-242 / 194–198)	Sucrose hydrolysis and transfructosylation
VII	PLI	PL(V/I)	330–332	**PLV**	(260-262 / 216–218)	Conserved among fructosyltransferases; unknown function
VIII	DEIER	D(Q/E)(T/I)ER	339–343	LK(I/V)(V/L)KD	(270-274 / 226–230)	Catalytic centre, acid–base catalyst, conserved in GH 68 family
IX	YLFT	YLF(T/S)	354–357	AILG	(285-288 / 241–244)	Conserved in microbial fructosyltransferases
X	YKPLN	YXP(L/M)N	388–392	**YK**GFQ	(363-367 / 319–323)	Conserved in GH 68 family
XI	TYS	TYS	410–412	D**YS**	(381-383 / 311–313)	Orientates nucleophilic residue for transfructosylation

In order to compare whether the motifs are more conserved in the *Bombella* FTases than in other GH 32 enzymes, an alignment was made with the enzymes mentioned above and the motifs were marked (Figure 27). Of the eleven motifs, some actually appear to be more conserved, such as motif I, IV and VII. Other motifs, such as V and VI, which are associated with polymerization / transfructosylation, do not show increased conservation in *Bombella* FTases.

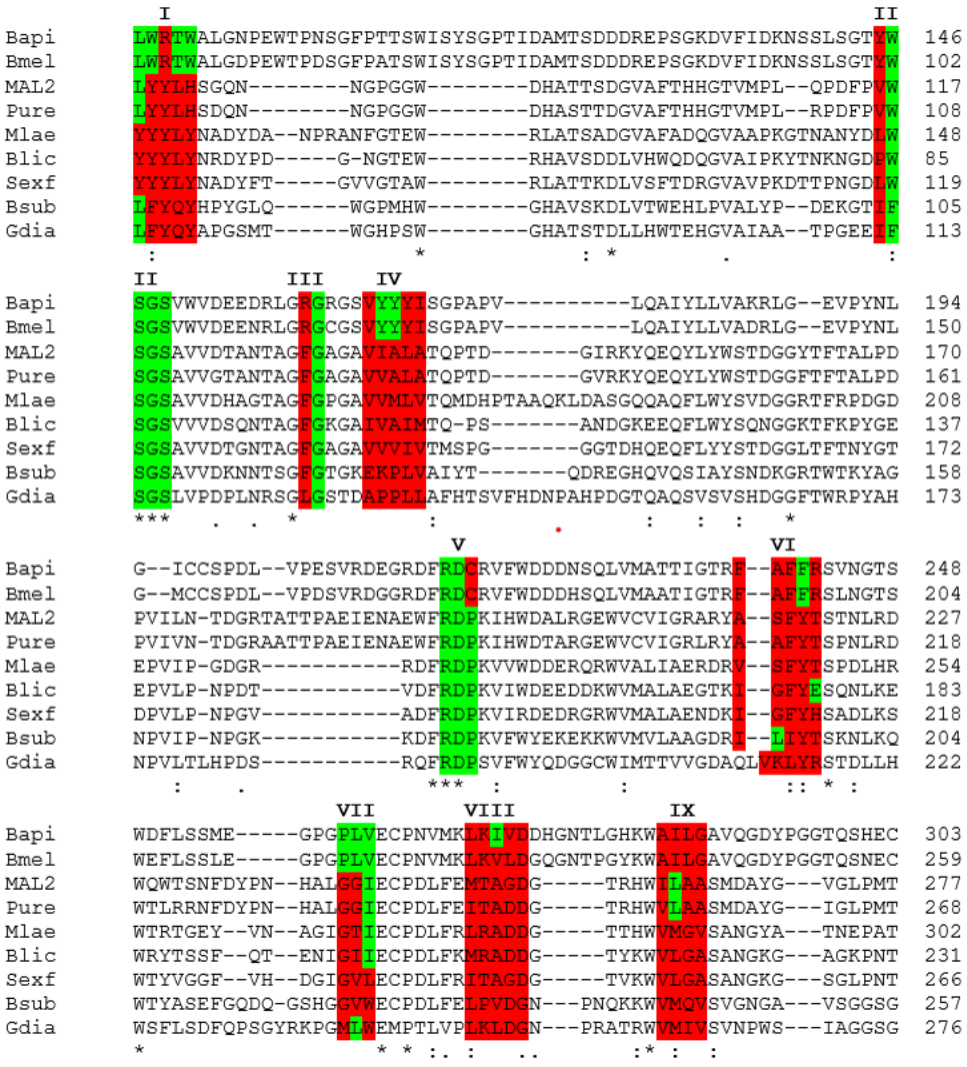


Figure 27: Alignment of GH 32 family enzymes. Letters with a green background correspond to the consensus sequence of conserved motifs of microbial fructosyltransferases (GH 68 family), red letters do not. Bapi = *Bombella apis* fructosyltransferase, Bmel = *Bombella mellum* fructosyltransferase, MAL2 = *Microbacterium* sp. AL-210 levan fructotransferase, Pure = *Paenarthrobacter ureafaciens* levan fructotransferase, Mlae = *Microbacterium laevaniformans* levanase, Blic = *Bacillus licheniformis* endolevanase, Sexf = *Streptomyces exfoliatus* levanbiohydrolase, Bsub = *Bacillus subtilis* levanase, Gdia = *Gluconacetobacter diazotrophicus* levanase.

4 Discussion

This work has provided new insights into the bacterial genus *Bombella*, which is associated with honey bee symbiosis. In the first part fundamental topics were dealt with. The taxonomy of the genus, which was previously unclear, was clarified by including all available genomes. This led to the realization that the genus is more diverse than previously described in the literature. To address this, new strains were isolated from the environment of the western honey bee *Apis mellifera* and phylogenetically analyzed. This resulted in the description of the four novel species *B. pluederhausensis*, *B. pollinis*, *B. saccharophila* and *B. dulcis*, including physiological characterization and differentiation from previously described species. Also, it was then possible to assign all genome data to validly described species. The valid taxonomic classification made it possible to analyze the differences between or within *Bombella* species using comparative genomic tools. The pentose phosphate pathway could be reconstructed for all strains, but all strains have incomplete glycolysis and an incomplete TCA cycle. Then, the response of a *Bombella* strain to glucose and NaCL stress was investigated at a proteomic level. This was of interest as all tested strains are tolerant to high glucose concentrations but relatively sensitive to NaCL. In both cases, various transporter-associated proteins were differentially expressed, but the general stress response was fundamentally different. In addition, the intracellular metabolites were examined under both stress conditions, whereby an accumulation of various molecules occurred primarily under glucose stress.

In the second part of the work, possible applications of strains of the *Bombella* genus were explored. A use as starter culture strains for the fermentation of fruit juices has been considered. A general suitability was determined in growth experiments, as all tested strains can grow in four different fruit juices. In addition, adaptation to the juice matrix by UV mutagenesis was successfully carried out. In a further proteomics experiment, the major difference between the wild-type strain and the adapted strain was found to be the reduced expression of flagella-associated proteins. Finally, it was investigated whether *Bombella* strains form extracellular polysaccharides. Two strains that form HMW fructans were identified. By heterologous expression, enzymes of the GH 32 family were identified, which catalyze the synthesis of the polymer from sucrose. In this section, these findings will be discussed and placed in the context of current scientific knowledge.

4.1 Phylogenetic analysis of the genus *Bombella* and revised taxonomy

Note: Parts of this section have already been published in Härer et al. (2022) and Härer et al. (2023b).

At the start of this work, the genus *Bombella* was represented by 4 different species. *B. intestini* was described in 2014 (Li et al. 2015), followed by *B. apis* (Yun et al. 2017), *B. favorum* and *B. mellum* (Hilgarth et al. 2021). Here, 22 *Bombella* genomes were analyzed phylogenetically. Without considering the type strains, all but three genomes were grouped with the species *B. apis*. The other three strains *Bombella* sp. AS1, *Bombella* sp. ESL0378 and *Bombella* sp. ESL0385 were identified as Bombella strains, but could not be assigned to any validly described species.

In recent developments, the genus *Parasaccharibacer* and individual strains of the genus *Saccharibacter* have been assigned to the genus *Bombella*, more precisely to the species *B.apis* (Smith et al. 2021). Although the genus *Parasaccharibacter* has been published before the genus *Bombella* (Corby-Harris et al. 2014; Li et al. 2015), the publication was not in accordance to IJSEM standards and therefore not valid. The affected strains *B. apis* A29, *B. apis* AM168, *B. apis* G773c, *B. apis* 3A1 and *B. apis* M18 were also considered in this work, confirming the reclassification. The work by Smith et al. (2021) also pointed out the potential affiliation of *Bombella* sp. AS1 to an undescribed species. The valid description (in accordance to IJSEM standards) of the novel species *B. pluederhausensis*, *B. pollinis*, *B. saccharophila* and *B. dulcis*, which was the result of this work, has made it possible to classify the strain as *B. saccharophila* AS1 (Härer et al. 2023b). In addition, the two other strains without affiliation could be classified as *B. pollinis* ESL0378 and *B. pollinis* ESL0385. This means that species assignment was possible for all *Bombella* genomes available online.

Of the 26 phylogenetically analyzed *Bombella* strains in this work, only three were not isolated from the environment of the western honey bee *Apis mellifera*. *Bombella apis* TMW 2.1886 and *Bombella apis* TMW 2.1888 were isolated from the european dark bee *Apis mellifera mellifera* and *Bombella intestini* R52487^{T} from the red-tailed bumble bee *Bombus lapidarius* (Härer et al. 2022; Li et al. 2015). None of the three strains is clearly phylogenetically separated from the *Apis mellifera* associated strains, arguing against specific evolutionary adaptation to the host. In the literature, Alpha 2.2 *Acetobacteraceae* are often mentioned in the context of honey bee microbiome studies, which represents strains from the genus *Bombella* (Corby-Harris et al. 2014; Hilgarth et al. 2021). It has already been described that this group occurs in various bee genera like

Caupolicana yarrow, *Xylocopa californica* or *Apis dorsata* (Martinson et al. 2011). Since bacteria of the *Bombella* genus, like *B. pollinis* TMW 2.2556, can be found in pollen samples, there could be an exchange of bacterial strains between different bee species when collecting nectar and pollen (Prado et al. 2022). This would be an explanation for why there was no host-specific phylogenetic grouping. Such horizontal transmissions have already been described for other AAB and their respective insect hosts (Gonella et al. 2012).

4.2 Comparative genomics in the light of beehive habitat adaption

Note: Parts of this section have already been published in Härer et al. (2022).

Once a taxonomic classification of all *Bombella* strains was possible, comparative genomic tools were applied in order to identify intra- and interspecies similarities and differences.

4.2.1 Predictive carbohydrate metabolism of *Bombella* spp.

The cytoplasmic carbohydrate metabolism of *Bombella* spp. was predicted by comparative genomic analysis (Figure 6) including the EMP pathway, the PPP and the TCA cycle. All genomes lack genes for phospho-fructokinases, disrupting the EMP pathway. Incomplete glycolysis was already described for several AAB such as *G. oxydans* and *Acetobacter pasteurianus* (Azuma et al. 2009; Illeghems et al. 2013; Prust et al. 2005).

It is assumed that intracellular glucose is mainly metabolized via the PPP or the Entner-Doudoroff (ED) pathway for which all genes were identified. This cytoplasmic carbon flux was verified for related *G. oxydans* 621H by a ^{13}C-based metabolic flux analysis (Hanke et al. 2013). Intracellular D-fructose can also be incorporated in this flux via a fructokinase.

Bombella genomes lack three or, in the case of *B. pollinis* and *B. saccharophila* strains, six genes of the TCA cycle. An incomplete TCA cycle missing three genes is described for other acetic acid bacteria (Mullins et al. 2008; Brown and Wernegreen 2019; Prust et al. 2005). In that case all steps until the synthesis of succinyl-CoA are present. To prevent an accumulation of TCA-cycle intermediates it is assumed that it is regulated to just meet the cellular demand of 2-oxoglutarate as a precursor for biosynthesis of the glutamate family of amino acids (Hanke et al. 2013). Other intermediates of the TCA cycle can derive from different sources, e.g. amino acids. For *Bombella intestini* theoretical pathways linked to L-asparagine were constructed that would result in succinate, fumarate or oxaloacetate (Li et al. 2016). No studies were found that dealt with acetic

acid bacteria missing six genes of the TCA cycle and hence further investigations would be necessary to elucidate cellular mechanisms.

Acetic acid bacteria are well known for their ability to incompletely oxidize sugars and alcohols in the periplasm in so called oxidative fermentations (Matsushita et al. 1994). The responsible membrane-bound DHs are coenzyme-dependent and coupled to the respiratory chain (Matsushita et al. 1994; Adachi et al. 2003). For *G. oxydans* it was shown that most of the glucose in the medium is not taken up by the cell but is oxidized in the periplasm (Hanke et al. 2013). Five membrane-bound DHs were identified in *Bombella* spp., namely a PQQ-dependent glucose DH, a gluconate-2 DH, a quinone-dependent dihydroorotate DH, a D-lactate DH and an ALDH gene with an undefined substrate spectrum. Prediction of the substrate spectrum is not expedient, since it was shown that a broad spectra of substrates are accepted in comparable enzymes (Matsushita et al. 2003). Previously a related enzyme was annotated as a glycerol, sorbitol and glycerol DH (Prust et al. 2005). Other identified electron donors of the respiratory chain were a type II NADH DH and a flavoprotein-ubiquinone oxidoreductase with unknown substrate spectrum. In contrast, *Gluconobater oxydans* possesses 32 membrane-bound DH (Prust et al. 2005; Richhardt et al. 2013). The reduced amount of membrane-bound DH in *Bombella* spp. and the focus on glucose/gluconate oxidation might be an indicator of the adaption to the honey bee environment, where glucose is constantly available (Simo and Christensen 1962; Siddiqui 1970).

4.2.2 Potential role of extracellular invertase in melezitose degradation

The “honeydew flow disease” in honey bees results from the feed on honeydew and impacts whole colonies. It is linked to the presence of the honeydew trisaccharide melezitose (Seeburger et al. 2020). Melezitose is hydrolyzed to some extent in the bee gut by enzymes with invertase activity, expressed either by the microbiome organisms or by the bee itself, but accumulation of the sugar could lead to severe symptoms (Seeburger et al. 2020). It was shown that *B. apis* was one of the microbiome bacteria that was not negatively influenced by a melezitose feed (Seeburger et al. 2020). This phenomenon might be linked to the expression of an extracellular invertase present in all *Bombella* spp. analyzed, which carries a twin-arginine translocation signal for secretion and a GH 32 motif (Supplementary Table S2). The catalytic activity of such enzymes in the bee environment might be crucial to prevent melezitose accumulation and the “honeydew flow disease” associated with it.

4.2.3 Potential antifungal properties

Bombella apis is associated with the protection of honey bee hives from fungal pathogens (Miller et al. 2021). It is presumed that an antifungal metabolite is responsible for the protective effect, probably synthesized by enzymes of a T1PKS gene cluster (Miller et al. 2021). All *Bombella* genomes contain such a cluster, but it was also identified in other acetic acid bacteria like *Saccharibacter floricola* DSM 15669, *Gluconacetobacter diazotrophicus* PA1 5 and *Asaia bogorensis* NBRC 16594. The wide distribution of the T1PKS gene cluster in other acetic acid bacteria indicates that it is not a genetic feature that is associated with the symbiosis of *Bombella* spp. and honey bees. It therefore also seems unlikely that these genes are associated with the antifungal properties of *Bombella* strains.

The antifugal properties of *Bombella apis* might not be caused by the synthesis of a secondary metabolite, but could also be a result of primary metabolism. For example, it would be possible that the extracellular accumulation of gluconate via oxidative fermentation or secretion of acetate could have an impact on fungal growth by lowering the pH value. Separate alterations of the surroundings could accumulate to the measured decrease in fungal growth.

4.2.4 Tetracycline resistance

The resistance of *Bombella apis* MRM1^T towards tetracycline and doxycycline was shown in a disk diffusion assay. Tetracycline is a broad-spectrum antibiotic used in non-EU countries to prevent microbial infections with e.g. *Paenibacillus larvae*, a bacteria responsible for foulbrood disease (Evans 2003). It was shown that tetracycline has negative effects on the size and composition of the honey bee gut microbiome and on the resistance of honey bees towards other opportunistic bacterial pathogens (Raymann et al. 2017).

The genomes of *Bombella* spp. were analyzed for genes associated with tetracycline resistance. No genes were found, except for a tetracycline resistance transcriptional repressor (*tetR*; IGM82_03615) and a tetracycline efflux MFS transporter (*tetG*; IGM82_03620) in *Bombella apis* MRM1^T. Both genes are located in proximity and the transcriptional repressor TetR likely regulates the expression of the efflux-MFS transporter dependent on the presence of an tetracycline antibiotic (Ramos et al. 2005). The most common mechanism of tetracycline resistance is active efflux of the drug, and numerous genetic determinants have been described. The *tetG* gene we identified in *Bombella* is most clearly related (95 % similarity) to the tetG of the Gram-negative sewage isolate

Paradevosia shaoguanensis J5-3T, which has also been described as tetracycline resistant (Geng et al. 2015).

When using antibiotics in beekeeping, it must be noted that the spread of resistance genes could be promoted. Also, the lacking resistance of the majority of *Bombella* spp. and of other organisms of the honey bee microbiome to antibiotics should be considered when using such agents for prevention purposes. It was shown that overtreatment with antibiotics interfered with normal microbiome function, rendering the host more susceptible to opportunistic and antibiotic resistant microbes (Anderson et al. 2023).

4.3 Glucose and NaCl tolerance of *Bombella* spp.

Physiological studies on all *Bombella* type strains have shown that they have a high tolerance to glucose, while a low tolerance to NaCl was observed (Table 9). All available strains were additionally tested for the maximal tolerated glucose concentration and compared to the industrial relevant *Gluconobacter oxydans* DSM46615. While growth up to 300 g/L glucose was observed for *G. oxydans*, all but one *Bombella* strain were able to grow up to 400 or 500 g/L glucose. For *B. favorum* TMW 2.1880, the glucose and NaCl concentrations were determined at which high stress acts on the cells, but growth was still measurable. Based on growth curves, the concentrations were set to 400 g/L glucose and 10 g/L NaCl. These concentrations correspond to 2.2 M glucose and 0.12 M NaCl. This difference is not surprising as honeycombs, a habitat of *Bombella* strains, contain up to 400 g/L glucose, while many honeys are considered sodium-free foods (Ball 2007).

High concentrations of salts or sugars in the environment of bacteria have a dehydration effect on the cells due to osmotic stress typically evaluated by the water activity (A_w), which indicates the concentration of freely mobile water molecules (Russell et al. 2003). Sugar solutions cause water activity largely by physical displacement of water molecules, whereas salt solutions decrease water activity mainly by binding water molecules (Kasaai 2014). Also, the same concentration of glucose or NaCl in the media does not result in the same A_w value, as shown in Table 15.

Table 15: A_w-values of media with different concentrations of glucose or NaCl (Fredsgaard et al. 2017).

	Concentration [g/L]	10	100	150	200	250	300	400	500	600
A_w	Glucose	0.98	-	-	-	-	0.96	0.94	0.91	0.9
	NaCl	0.95	0.92	0.88	0.85	0.8	0.76	-	-	-

Based on Table 15 it can be assumed that the A_w-values at 400 g/L glucose and 10 g/L NaCl are similar, which correlates with the determined concentrations that caused similar stress levels on *B. favorum* TMW 2.1880. However, since many *Bombella* strains grow up to 500 g/L glucose (A_w-value 0.91), but no strain can grow above 10 g/L NaCl (A_w-value 0.95), there does not seem to be a clear correlation between growth inhibition and A_w-value. In contrast to *Bombella* strains, bacteria isolated from high-salt environments were shown to tolerate lower A_w-value in NaCl media than in glucose media (Fredsgaard et al. 2017). The cellular adaptations to the natural habitat therefore seem to have a great influence on the tolerance to NaCl or glucose.

4.3.1 Cellular stress mechanisms in *B. favorum* TMW 2.1880

Proteomic studies were performed with *B. favorum* TMW 2.1880 exposed to glucose and NaCl stress. Over a cultivation time of 27 h the expression levels of detectable proteins were monitored in media containing 400 g/L glucose or 10 g/L NaCl. A reference cultivation without osmotic stress was grown in LMG 404 media.

Nine different transporter associated proteins were differentially expressed under glucose or NaCl stress. A common response of bacteria to osmotic stress is the active adjustment of the distribution of selected solutes across the cytoplasmic membrane to adjust the turgor pressure (Wood 1999). An example is the ProP transporter *in E. coli*, which acts as an osmosensor and can regulate osmotic pressure by pumping the amino acid proline into the cytoplasm. The transporter activates when dehydration, i.e. a low A_w-value, is detected (Racher et al. 1999; Culham et al. 2003). In *Gluconacetobacter diazotrophicus,* a downregulation of various passive transporters was detected in proteomic studies, which is presumably preventing the penetration of extracellular molecules into the cytoplasm (Leandro et al. 2021). Transporters can therefore have both positive and negative influences on the cell under osmotic pressure, which would explain why both up- and downregulations were detected for *B. favorum* TMW 2.1880 under glucose or NaCl stress.

Three different cytosolic alcohol dehydrogenases showed increased expression under glucose stress. From the protein sequence, it is not possible to determine the substrate spectrum. Since one protein was annotated as a mannitol DH and one as a butanol DH, it can be assumed that intracellular oxidoreductase reactions with carbohydrates as substrates are catalyzed. For *Gluconobacter oxydans* it has been described that a mannitol DH is a key enzyme of osmoprotection, by catalyzing the intracellular formation of mannitol from mannose (Zahid et al. 2015; Zahid and Deppenmeier 2016). The intracellular accumulation of so called compatible solutes to balance the osmotic pressure is a common phenomenon that is described for many prokaryotes (Empadinhas and Da Costa 2008). The used compounds can be sugar alcohols like mannitol, but also amino acids, sugars or other osmotically active molecules (Killham and Firestone 1984; Elbein et al. 2003; Galinski 1995; Curatti et al. 2000). It is therefore possible that all or some of the three ALDHs from *B. favorum* TMW 2.1880 are associated with the formation of compatible solutes.

This hypothesis is supported by the metabolomics data (Section 3.6.4): two sugar alcohols with the molecular formulas $C_5H_{12}O_5$ and $C_4H_{10}O_4$ could be products of the alcohols DHs and were significantly more abundant under glucose stress. The molecular formula $C_5H_{12}O_5$ corresponds to the sugar alcohols arabitol, xylitol or ribitol. Arabitol and xylitol have already been described as important compatible solutes in yeasts (Abadias et al. 2000; Chirife et al. 1984). Since no significant difference was found when comparing the NaCL stress medium with the reference medium, there could be a connection with the ALDH CPA57_07465, which was only increasingly expressed under glucose stress. Other sugar alcohols with the molecular formula $C_4H_{10}O_4$ (erythritol or threitol) were most abundant under glucose stress, but also increased under NaCL stress compared to the reference. Descriptions of the use of erythritol as a compatible solute in yeasts can also be found in the literature (Hallsworth and Magan 1995; Yang et al. 2015). There are also several descriptions of bacteria that use sugar alcohols as compatible solutes (Kets et al. 1996; Loos et al. 1994; Zahid et al. 2015).

The molecular formulas $C_6H_{12}O_6$ and $C_5H_{10}O_5$ most likely correspond to pentose or hexose monosaccharides. These molecules are significantly the most abundant in *B. favorum* TMW 2.1880 under glucose stress, but also increased under NaCL stress compared to the reference. Both could theoretically also serve as compatible solutes, but no literature was found where these molecules act as compatible solutes in microorganisms. $C_6H_{12}O_6$ could represent glucose, which is present in the cultivation media. Particularly with glucose stress media, it can be assumed that glucose enters

the cytoplasm. However, since it is also increased under NaCl stress, it may be required intracellularly as a precursor for the formation of compatible solutes, which could also be the case for $C_5H_{10}O_5$ molecules.

Another protein that showed increased expression in *B. favorum* TMW 2.1880 under glucose stress was the molecular chaperone CPA57_04705. Molecular chaperons assist the correct non-covalent assembly of proteins, but are not components of these proteins when they are performing their normal biological functions (Ellis 1993). They are typical heat-shock proteins, which means that they are known to be expressed under stress conditions, such as osmotic stress (Lund 2001; Yang et al. 2006; Sugimoto et al. 2008).

The protein CPA57_08255 was expressed most under glucose stress, but also showed higher expression under NaCl than in the reference. It annotated as a pyrroline-5-carboxylate reductase, an enzyme that catalyzes the formation of proline from 1-pyrroline-5-carboxylate, which is the last step of the proline metabolism. Proline is known to act as a compatible solute in other bacteria (Killham and Firestone 1984; Galinski 1995). Again, the proteomics data is supported by the metabolomics data, which shows that proline is most abundant in the glucose stress sample, followed by the NaCl stress sample and the reference. However, it is also possible that part of the measured proline was not *de novo* synthesized, but actively taken up from the medium by one of the up-regulated transporters as it is described for the *E. coli* transporter ProP (Culham et al. 2003; Racher et al. 1999). This mechanism could be an adaption to the natural environment of *Bombella* strains, since proline is the predominant free amino acid of honey and is also found in nectar (Iglesias et al. 2004; Ball 2007).

NaCl stress induced the expression of proteins that are associated with the formation of a T4SS complex. Nine proteins that should form the subunits VirV4, VirB7, VirB8, VirB9, VirB10, VirB11 and VirD4 were detected two hours after inoculation of the NaCl stress media and the expression level remained at a high level throughout the cultivation. VirB6 was detected at 4, 12 and 27 h at low expression levels. The genes of the subunits VirB1, VirB2, VirB3, and VirB5 were identified in the genome, but the proteins were not detected in the proteomics data. The reason why not all of the T4SS proteins were detected could be due to low expression, subcellular localization or due to a technical reason. A schematic structure of T4SSs is shown in Figure 28 (Grohmann et al. 2018).

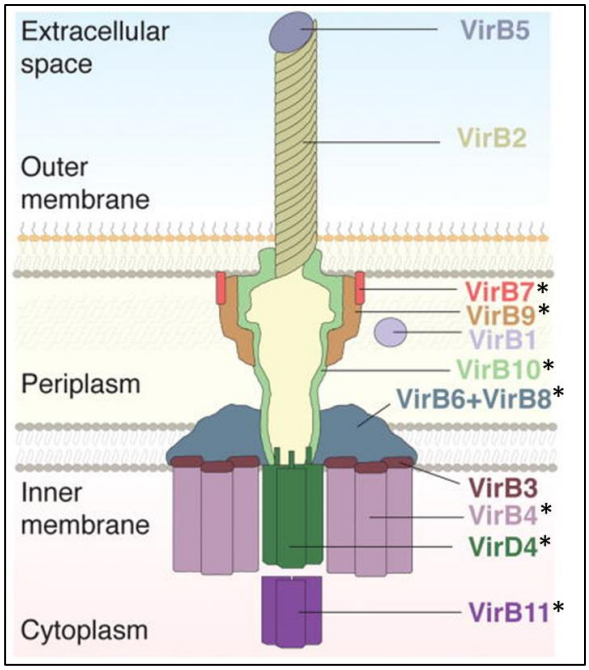


Figure 28: Schematic structure of Type IV Secretion Systems including all subunits (Grohmann et al. 2018). Subunits marked with * were upregulated in *B. favorum* TMW 2.1880 under NaCl stress.

The complex structure and interaction of the individual proteins allows the cell to transport macromolecules from the cytoplasm directly to other cells (Christie and Vogel 2000). T4SSs can have a variety of cellular functions: recognition and translocation of single-stranded DNA substrates (conjugation) to bacterial receptors, delivery of effector proteins to eukaryotic target cells, DNA exchange with the environment, contribution to biofilm development, and delivery of a killing toxin to bacterial neighbors (Grohmann et al. 2018). Type IV pili can even be involved in cell motility (Wall and Kaiser 1999; Merz et al. 2000). Unfortunately, why this system is expressed under NaCl stress in *B. favorum* TMW 2.1880 and what cellular function it performs could not be clarified.

Another effect of NaCl stress on *B. favorum* TMW 2.1880 cells was the reduced expression of proteins associated with flagella. The corresponding genes are arranged on an operon with a total of 75 ORFs. Flagella are locomotive organelles for bacterial motility. They consist of a basal body, which acts as a reversible rotary motor, a hook, which acts as a universal joint, and a filament, which acts as a helical screw (Morimoto and Minamino 2014). The schematic structure of a bacterial flagella is shown in Figure 29.

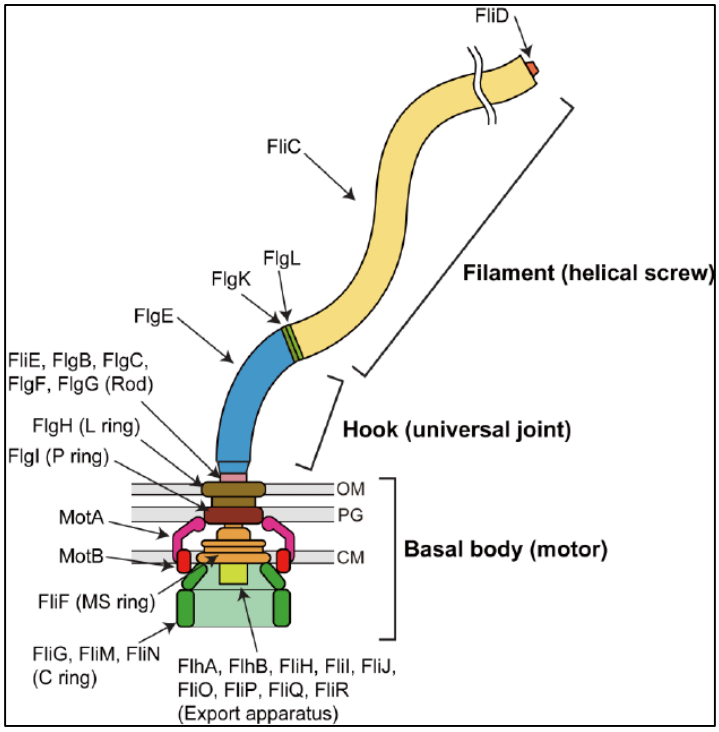


Figure 29: Schematic diagram of a bacterial flagellar motor (Morimoto and Minamino 2014). OM: outer membrane; PG: peptidoglycan layer; CM: cytoplasmic membrane.

For *Escherichia coli*, it has already been described that high NaCl concentration and other cellular stress phenomena such as high pressure lead to the loss of flagella protein (Li et al. 1993). The authors hypothesize that energy conservation due to the lack of expression is a survival mechanism of the cells. Since the energy for flagellar motor rotation is supplied by the electrochemical potential of specific ions (e.g. Na+) across the cytoplasmic membrane (Ravid and Eisenbach 1984; Manson et al. 1977), it could also be hypothesized that a high NaCl concentration in the media could impair the functionality of the flagellar system and thus a regulatory mechanism takes effect.

4.3.2 Summary - cellular response to glucose or NaCl stress

Proteomics experiments were performed in combination with metabolomics experiments to elucidate stress mechanisms in *B. favorum* TMW 2.1880 under glucose or NaCl stress. Even though glucose and NaCl lower the A_w-value of the media and thus exert an osmotic pressure on the cells, different cell responses were observed.

In both cases, various membrane-bound transporter proteins were up- or downregulated. However, the transporters affected were different. Under osmotic stress, transporter proteins can have beneficial properties that are linked to the active equilibration of turgor pressure. Other, passive transporters, must be down-regulated to prevent molecules from entering the cytoplasm. The fact

that different transporters are involved in the glucose or NaCl stress response seems logical due to the different nature of the molecules.

Under glucose stress, mainly enzymes associated with the intracellular formation of compatible solutes were relatively up-regulated. The intracellular accumulation of sugar alcohols and proline was verified by metabolomics experiments and is in support of the proteomics data. Such an accumulation could also be measured in the cells under NaCl stress, but not for all of the molecules and to a significantly lesser extent than under glucose stress.

In contrast to glucose stress, under which individual enzymes were affected, two complete operons were differentially regulated under NaCl stress. The expression of a T4SS operon was induced. However, the influence of this system on the survival of the cell could not be clarified. Proteins that are associated with the formation of a flagella and are genetically arranged on an operon were down-regulated under NaCl stress. Here too, no clear connection to the reduced A_w-value could be made. However, it is it is possible that the flagella are no longer functional due to the increased Na+ concentration, leading to down-regulation.

As described at the beginning of this section, *Bombella* strains can persist at lower A_w-values if they have been lowered by glucose and not by NaCl. As shown here, the cellular response to glucose and NaCl stress was different in *B. favorum* TMW 2.1880. Under glucose stress, individual proteins were regulated that were connected to the regulation of osmotic pressure by accumulating compatible solutes. These mechanisms were also observed in the cells grown in NaCl stress media, but to a lesser extent. In addition, two operons with a total of over 80 genes were regulated differently under NaCl stress. It is assumed that the more specific stress response to glucose is linked to adaptation to the bacteria's beehive habitat, where cells are under constant osmotic stress due to high sugar concentrations.

4.4 *Bombella* spp. as starter cultures for fruit juice fermentation

While natural fruit juice consumption is declining in Germany (Figure 3) interest in fermented beverages such as kombucha is growing globally (Kim and Adhikari 2020). In this work, the suitability of strains of the genus *Bombella* for the development of novel beverages through the fermentation of fruit juices was investigated. For this purpose, it was tested whether available strains could grow in grape juice, apple juice, sour cherry nectar or orange juice from concentrate. Growth was determined by measuring the cell count after 7 and 24 h. In general, an increase in cell counts was measured for all strains, i.e. growth was detected. In some cases, however, a drop in cell counts was measured between 7 and 24 h, meaning that the cultures were already in the death phase.

Fruit juices have several microbiological barriers that should inhibit the growth of most bacteria. For example, the fruit juices used have acidic pH values between 3.3 and 3.8 and high total sugar concentrations between 110 and 170 g/L. The physiological characterization of the *Bombella* type strains revealed a growth limit between pH 3 and 3.5. The death phase could therefore have been initiated by further acidification of the juices. The sugar concentrations, on the other hand, should not have presented a hurdle. Furthermore, plant polyphenols can have an inhibiting effect on the growth of bacteria (García-Ruiz et al. 2011; Sakanaka et al. 2000; Tabasco et al. 2011). The polyphenols from the fruits are also present in the corresponding juices. The concentration (total polyphenols) should be highest in cherry juice and lowest in orange juice (Díaz-García et al. 2013). Perhaps this is the reason for the strong growth of *Bombella* strains in the orange juice fermentations compared to the other juices.

Since the growth of all *Bombella* strains in different fruit juices has been demonstrated, a general suitability for fermentation can be established. The taste of the novel beverages was tested in a parallel project, but unfortunately no results were available at the time this work was completed.

4.4.1 Adaptability of *B. favorum* TMW 2.1880 to the fruit juice matrix

It was investigated whether *B. favorum* TMW 2.1880 could be adapted to grape juice concentrate. However, the adaptation was not carried out directly in grape juice concentrate, but in a medium containing 400 g/L glucose. Adaptation to a single stressor (sugar stress) was chosen to facilitate the interpretation of the cellular changes, which were later studied in a proteomics experiment. For the adaption, a culture was treated with UV light for 10 min, and then used to inoculate fresh

medium with 400 g/L glucose. After incubation for 24 h the process was repeated. This was repeated 50 times in total. The adapted strain showed improved growth behavior glucose concentration from 100 to 400 g/L glucose, but the tolerated glucose concentration was not increased. When grown in grape juice concentrate, the adapted strain showed improved growth at all total sugar concentrations and was even able to grow in concentrate with 400 g/L total sugar where the wild-type strain failed to grow.

Adaptation to an external factor through the constant influence of this factor over many generations is also referred to as adaptive laboratory evolution (Dragosits and Mattanovich 2013). This allowed the adaption of bacteria to many conditions, such as higher ethanol tolerance or growth at higher temperatures (Goodarzi et al. 2010; Rudolph et al. 2010). However, this is the first time that successful adaptation to the fruit juice matrix has been demonstrated.

4.4.2 Proteomic changes in the adapted strain

The *B. favorum* TMW 2.1880 strain that was adapted for 50 generations in glucose stress medium and the wild-type strain were compared in a proteomics experiment. All significantly differentially expressed proteins were assigned to a functional group based on COGs. This allowed identification of the cellular functions that led to improved growth at high glucose concentrations (400 g/L) and in grape juice concentrate (20 °Brix / 200 g/L total sugar). In both cases, there was a strong overlap in the differentially expressed proteins (65 %), so similar mechanisms appear to be leading to the altered growth. The most relevant groups were "Signal transduction mechanisms", "Cell wall/membrane/envelope biogenesis" and "Cell motility". However, closer analysis revealed that most of these proteins are involved in the regulation, structure and function of flagella. In addition, these proteins are all located on the previously discussed operon that was down-regulated under NaCl stress. It has been hypothesized that the improved growth is due to energy conservation resulting from the absence of flagella. Calculations for *E. coli* showed that the assembly of flagella consumes 5.0 % and the operation of the flagella another 5.2 % of the total energy of the cells, i.e. a total of 10.2 % (Schavemaker and Lynch 2022). If this energy is used for other cellular processes necessary for growth, such as cell wall synthesis, the improved growth behavior could be explained.

4.5 Fructan-producing *Bombella* strains

Note: Parts of this section have already been published in Härer et al. (2023a).

Out of 12 Bombella strains tested only *B. mellum* TMW 2.1889 and *B. apis* TMW 2.1884 produced EPS when grown on agar plates containing 50 g/L sucrose. The EPS were identified as pure fructans.

4.5.1 Fructan structure

Glycosidic linkages of the native *Bombella* fructans were determined by methylation analysis. Both fructans possess 1,2-linked Fru*f* units in addition to 2,6- and 1,2,6-linked Fru*f* units. Typically, bacterial fructans are classified into levans and inulins, which only contain one linkage type. The fructofuranose (Fru*f*) backbones of levans have *β*-(2→6) linkages and inulins are composed of *β*-(2→1) linkages (Velázquez-Hernández et al. 2009). Inulins are common in plants, but their formation is only associated with a few Gram-positive genera (van Hijum et al. 2002; Velázquez-Hernández et al. 2009; Ni et al. 2019; Xu et al. 2019). Levans on the other hand are produced by a variety of prokaryotes such as lactic acid bacteria (Ni et al. 2018), pseudomonads (Visnapuu et al. 2011) and acetic acid bacteria (Jakob et al. 2019). Bacterial mixed-linkage fructans have not been described in literature yet. However, in the plant kingdom fructans containing 1,2- and 2,6-linkages, so called graminans, are known (Verspreet et al. 2014).

AF4-MALLS was used to determine the molecular weight of the recovered fructans from *Bombella*. The average molecular weight of the *B. apis* TMW 2.1184 and *B. mellum* TMW 2.1189 fructan was 77.6 mDa (± 0.2 %) and 80.5 mDa (± 0.5 %), respectively. Levans from other acetic acid bacteria such as *Gluconobacter frateurii* or *Neoasaia chiangmaiensis* have also been measured with an AF4-MALLS system. The molecular weight varied between 4 and 2,000 mDa, a range in which also the *Bombella* fructans lie (Jakob et al. 2013).

4.5.2 *Bombella* FTases in comparison with other GH 32 and GH 68 family enzymes

Two ORFs were identified in *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889 genomes that possibly code for enzymes with FTase activity. A similar ORF was also identified in a third strain that did not produce slime on agar plates with sucrose. However, the gene sequence contained a missing base, which most likely resulted in a frame shift and a non-functional enzyme. To verify the FTase activity of the enzymes, the corresponding genes were heterologously expressed in *E. coli* Top10. The *E. coli* colonies expressing the enzyme from *B. apis* TMW 2.1884 produced

slime on agar plates containing sucrose, which allowed the enzymes to be assigned to the production of EPS. More detailed analyses showed that the EPS are fructans with almost identical glycosidic linkages to the native fructan. The enzymes identified are therefore almost certainly responsible for the fructan formation in *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889. Phylogenetic and structural analysis of the peptide sequences assigned the *Bombella* FTases to the GH 32 family.

Typically, the extracellular formation of fructans from sucrose is catalyzed by either inulosucrases (inulin; EC 2.4.1.9) or levansucrases (levan; EC 2.4.1.10). Both FTases are classified as hydrolase GH 68 family enzymes by the "Carbohydrate Active Enzyme Database". Instead of a hydrolysis reaction, where water is the acceptor of the fructosyl unit, they catalyze the transfer of the fructosyl unit of sucrose to another saccharide, which leads to the formation of a fructan and the release of glucose (Alamäe et al. 2023; Lammens et al. 2009; Martínez-Fleites et al. 2005; Meng and Fütterer 2003). All GH 68 family enzymes contain a five-bladed β-propeller structure harboring the catalytic triad, which consists of three acidic residues (Pons et al. 2004). They share this structure as well as a retaining reaction mechanism with enzymes of the GH 32 family which also belong to the GH family clan J (Pons et al. 2004; Alamäe et al. 2023). Unlike GH 68 family members, enzymes belonging to the GH 32 family generally have an additional C-terminal domain consisting of two six-stranded *β*-sheets, which are composed of antiparallel *β*-strands forming a sandwich-like fold (Lammens et al. 2009), which was also identified in the *Bombella* FTases.

Besides the differences in structure, the catalyzed reaction of GH 32 and GH 68 family enzymes is also different: Most identified and characterized enzymes of the GH 32 family catalyze hydrolysis reactions including a water molecule as an acceptor, for example the cleavage of sucrose into glucose and fructose (Liebl et al. 1998) or the degradation of levan into smaller molecules (Zhang et al. 2019). To our knowledge, there is no other description of bacterial enzymes of the GH 32 family involved in the formation of HMW fructans.

All protein motifs found in the FTases of *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889 that have been associated with fructan formation (Velázquez-Hernández et al. 2009) lie in the *N*-terminal five-bladed *β*-propeller catalytic domain region shared by all GH 32 and GH 68 enzymes. However, since these motifs of the *Bombella* FTases do not exactly correspond to the given consensus sequences, their actual function cannot be determined with certainty. In order to clarify the actual influence of the corresponding motifs on the formation of HMW fructans,

complex mutation studies would have to be carried out, as has already been done by Meng and Fütterer (2003) or Xu et al. (2022) for GH 68 family enzymes.

4.5.3 Future prospects

Apart from the open questions concerning fructan structures and catalysis mechanisms discussed in the previous sections, the discovery of the *Bombella* FTases has opened up numerous further research topics. A more detailed investigation of the heterologously expressed enzyme with regards to substrate specificity and enzyme kinetics could shed light on the actual function of the enzyme in *Bombella* strains possessing it. In addition, this could lead to the identification of potential applications. One application of the fructans, which can already be discussed, is the use as prebiotics in foods or in infant formulas (Kaur and Gupta 2002; Coussement 1999). Due to the uniqueness of the three different types of glycosidic linkages, *endo*-levanase hydrolysates of *Bombella* fructans should contain a variety of low molecular weight fructans and fructooligosaccharides and could therefore be particularly suitable as prebiotic food supplements.

5 Summary

The focus of this work was to investigate acetic acid bacteria of the genus *Bombella*, which are symbionts of the western honey bee *Apis mellifera*. The first part dealt with fundamental research topics. In order to clarify the previously unclear taxonomy and phylogeny, the genome data of all genomes available online and in our strain collection were analyzed. The phylogenetic analysis did not reveal any separation of strains from different isolation hosts. This means that an evolutionary adaptation to the different hosts is unlikely. Acetic acid bacteria are known for horizontal transmission, i.e. the exchange of strains between different hosts. Such horizontal transmission of *Bombella* strains therefore seems plausible, especially since all hosts are pollinators, i.e. an exchange can take place at flowers. Taxonomically, all strains, with the exception of the type strains, could be assigned to the genus *Bombella apis*. However, three strains could be assigned to the genus *Bombella*, but not to any validly described species. This shows that the diversity of the genus is greater than previously described in the literature.

In order to achieve a better coverage of the genus *Bombella*, new strains were isolated from the honey bee environment and characterized phylogenetically. Four novel species were described: *Bombella pluederhausensis*, *Bombella pollinis*, *Bombella saccharophila* and *Bombella dulcis*. As a result of the valid new description of the four species, the previously unassignable genomes could also be assigned to a species. The physiological investigations showed that the novel species behave similarly to the previously described species. All are mesophilic, tolerate low pH values, high glucose concentrations, but only low NaCl concentrations.

By comparative genomics, the metabolic pathways of the carbohydrate metabolism of the different species could be reconstructed. As with other acetic acid bacteria, both EMP pathway (glycolysis) and the TCA cycle are incomplete in all strains. In the strains of the species *B. pollinis* and *B. saccharophila* even six instead of three enzymes of the TCA cycle are missing, which has not been described before. The main pathway of glucose degradation appears to be via the pentose-phoshpate pathway, which has already been described for other acetic acid bacteria. The membrane-bound dehydrogenases, which are a typical trait of acetic acid bacteria, were identified in the *Bombella* genomes. No significant differences between the species could be detected. However, compared to other acetic acid bacteria, such as *Gluconobacter oxydans*, only relatively few membrane-bound dehydrogenases could be identified. It is assumed that this is an adaptation

to the habitat of the bacteria, in which glucose is constantly available, i.e. there is no dependence on different substrates.

Further genome analyses revealed that a strain of the species *B. apis*, which was isolated in South Korea, has resistance genes to the antibiotic tetracycline. Laboratory tests confirmed this resistance. Tetracycline is an antibiotic that is used by beekeepers outside the EU as a preventive measure in beehives. This is therefore probably another case of man-made spread of antibiotic resistance.

Physiological tests with available strains have shown that *Bombella* spp. have a high glucose tolerance. Most strains grew in media containing up to 500 g/L glucose. By combining a genomics and a metabolomics analysis, the cellular stress mechanisms of *B. favorum* TMW 2.1880 towards high glucose concentrations were investigated. In addition, cellular mechanisms under NaCl stress were investigated, as all strains studied showed comparable sensitivity to NaCl. Under glucose stress, which is to be expected in the natural habitat of the bacteria, individual proteins were expressed differently. Most of them were transporters or cytoplasmic dehydrogenases, which probably regulate the osmotic pressure, e.g. by forming compatible solutes. This intracellular accumulation of sugar alcohols and the amino acid proline to equalize osmotic pressure was confirmed by the metabolomics results. Similar mechanisms were also observed under NaCl stress, but weaker than under glucose stress. In addition, the expression of a complex type IV secretion system was induced and the expression of flagellar proteins was suppressed. The cellular responses to NaCl stress appear to be less targeted than to glucose stress. This is probably due to natural adaptations of the bacteria to their sugar-rich habitat.

In the second part of the work, possible applications of *Bombella* spp. were investigated. A use of the strains for the fermentation of fruit juices was considered, as the bacteria tolerate high sugar concentrations, can form extracellular food acids and other acetic acid bacteria are already used in the beverage industry. A resulting beverage could be marketed as calorie-reduced and natural. To test the basic suitability, the growth of the available strains was examined in four commercial fruit juices. All strains grew in all fruit juices despite high sugar concentrations, low pH values and plant polyphenols with antimicrobial properties. The next step was to investigate whether better growth of *B. favorum* TMW 2.1880 in the fruit juice milieu could be achieved by UV mutagenesis. Adaptation to a high glucose concentration, i.e. to a single stressor, resulted in a strain with improved growth properties in grape juice concentrate. Proteomics experiments were used to investigate which cellular functions were affected by the mutagenesis. It was found that the improved growth is probably not due to an adaptation to high glucose concentrations, but to the

loss of flagella, i.e. cell motility. The energy saved in this way can probably be used in other growth processes. The cells therefore appear to have adapted their energy consumption to the cultivation in shake flasks.

Acetic acid bacteria are also used in industry because of their ability to form extracellular polysaccharides (EPS). Since there have been no previous studies on EPS formation by *Bombella* spp. the EPS formation of the available strains was investigated on agar plates containing sucrose. Of the 12 strains from 8 different *Bombella* species, only *B. apis* TMW 2.1884 and *B. mellum* TMW 2.1889 formed EPS. Both EPS were identified as high molecular weight polymers (106-107 Da) by AF4-MALLS/UV analyses. External monosaccharide analysis via TFA hydrolysis showed that both EPS are fructans. The determination of glycosidic linkages by methylation analysis, also performed externally, revealed mainly 2,6-linked fructofuranose units (*Fruf*) with additional 1,2-linked *Fruf* units (10 %) and branched 1,2,6-Fruf units (7 %). No genes of enzymes of the glycoside hydrolase family (GH) 68, which are typically associated with the formation of high molecular weight fructans in bacteria, could be identified in the corresponding genomes. By heterologous expression in *E. coli* Top10, enzymes of the GH 32 family could be associated with the catalysis of fructan formation. The identified fructosyltransferases could be clearly distinguished phylogenetically and structurally from other previously described bacterial fructosyltransferases. These results represent the first description of bacterial enzymes of the GH 32 family that are involved in the formation of high molecular weight fructans.

6 Zusammenfassung

Diese Arbeit hat sich mit Essigsäurebakterien der Gattung *Bombella* befasst, welche Symbionten der westlichen Honigbiene *Apis mellifera* sind. Im ersten Teil wurden grundlegende Forschungsthemen behandelt. Um die bisher unklare Taxonomie und Phylogenie zu klären, wurden die Genomdaten aller online und in unserer lehrstuhleigenen Stammsammlung verfügbaren Genome ausgewertet. Bei der phylogenetischen Analyse konnte keine Trennung von Stämmen mit unterschiedlichen Isolationswirten festgestellt werden. Das bedeutet, dass eine evolutionäre Anpassung an die verschiedenen Wirte unwahrscheinlich ist. Essigsäurebakterien sind bekannt für horizontale Transmission, d.h. den Austausch von Stämmen zwischen verschiedenen Wirten. Eine solche horizontale Transmission von *Bombella* Stämmen erscheint daher plausibel, zumal alle Wirte Bestäuber sind, also ein Austausch an den Blüten stattfinden kann. Taxonomisch konnten alle Stämme, mit Ausnahme der Typstämme, der Gattung *Bombella apis* zugeordnet werden. Allerdings konnten drei Stämme zwar der Gattung *Bombella*, aber keiner validen beschriebenen Spezies zugeordnet werden. Dies zeigt, dass die Diversität der Gattung größer ist als bisher in der Literatur beschrieben.

Um eine bessere Abdeckung der Gattung *Bombella* zu erreichen, wurden neue Stämme aus dem Umfeld der Honigbiene isoliert und phylogenetisch charakterisiert. Dabei konnten vier neue Spezies beschrieben werden: *Bombella pluederhausensis*, *Bombella pollinis*, *Bombella saccharophila* und *Bombella dulcis.* Durch die valide Neubeschreibung der vier Spezies konnten auch die bisher nicht zuordenbaren Genome einer Spezies zugeordnet werden. Die physiologischen Untersuchungen zeigten, dass sich die neuen Spezies ähnlich wie die bisher beschriebenen Spezies verhalten. Alle sind mesophil, tolerieren niedrige pH-Werte, hohe Glucosekonzentrationen, aber nur geringe NaCl-Konzentrationen.

Durch vergleichende Genomik konnten die Stoffwechselwege des Kohlenstoffmetabolismus der verschiedenen Spezies rekonstruiert werden. Wie bei anderen Essigsäurebakterien sind sowohl die Glykolyse als auch der TCA-Zyklus bei allen Stämmen unvollständig. Bei den Stämmen der Spezies *B. pollinis* und *B. saccharophila* fehlen sogar sechs statt drei Enzymen des TCA-Zyklus, was bisher noch nicht beschrieben wurde. Der Hauptweg des Glucoseabbaus scheint über den Pentose-Phoshpat-Weg zu verlaufen, was bereits für andere Essigsäurebakterien beschrieben wurde. Die für Essigsäurebakterien typischen membranständigen Dehydrogenasen wurden in den *Bombella* Genomen identifiziert, wobei keine signifikanten Unterschiede zwischen den Spezies

festgestellt werden konnten. Im Vergleich zu anderen Essigsäurebakterien, wie z.B. *Gluconobacter oxydans*, konnten jedoch nur relativ wenige membranständige Dehydrogenasen identifiziert werden. Es wird vermutet, dass dies eine Anpassung an den Lebensraum der Bakterien darstellt, in welchem Glucose ständig verfügbar ist, also keine Abhängigkeit von verschiedenen Substraten besteht.

Weitere Genomanalysen ergaben, dass ein Stamm der Spezies *B. apis*, welcher in Südkorea isoliert wurde, Resistenzgene gegen das Antibiotikum Tetrazyklin besitzt. Laborversuche bestätigten diese Resistenz. Tetrazyklin ist ein Antibiotikum, das außerhalb der EU von Imkern vorbeugend in Bienenstöcken eingesetzt wird. Es handelt sich also wahrscheinlich um einen weiteren Fall der menschengemachten Verbreitung von Antibiotikaresistenzen.

Physiologische Tests mit verfügbaren Stämmen haben gezeigt, dass *Bombella* spp. eine hohe Glukosetoleranz aufweisen. Die meisten Stämme wuchsen in Medien mit bis zu 500 g/L Glucose. Durch die Kombination eines Genomik- und eines Metabolomik-Experiments wurden die zellulären Stressmechanismen von *B. favorum* TMW 2.1880 gegenüber hohen Glukosekonzentrationen untersucht. Zusätzlich wurden zelluläre Mechanismen unter NaCl-Stress untersucht, da alle untersuchten Stämme eine vergleichbare Sensitivität gegenüber NaCl aufwiesen. Unter Glucose-Stress, wie er im natürlichen Lebensraum der Bakterien zu erwarten ist, wurden einzelne Proteine unterschiedlich exprimiert. Bei den meisten handelte es sich um Transporter oder cytoplasmatische Dehydrogenasen, die wahrscheinlich den osmotischen Druck regulieren, z. B. durch die Bildung kompatibler Solute. Diese intrazelluläre Akkumulation von Zuckeralkoholen und der Aminosäure Prolin um den osmotischen Druck auszugleichen konnte durch Metabolomik-Experimente bestätigt werden. Ähnliche Mechanismen wurden auch unter NaCl-Stress beobachtet, jedoch schwächer als unter Glucose-Stress. Zusätzlich wurde die Expression eines komplexen Typ-IV-Sekretionssystems induziert und die Expression von Flagellenproteinen unterdrückt. Die zellulären Reaktionen auf NaCl-Stress scheinen weniger zielgerichtet zu sein als auf Glucose-Stress. Dies ist wahrscheinlich auf natürliche Anpassungen der Bakterien an ihren zuckerreichen Lebensraum zurückzuführen.

Im zweiten Teil der Arbeit wurden mögliche Anwendungen von *Bombella* spp. untersucht. Eine Verwendung der Stämme für die Fermentation von Fruchtsäften wurde in Betracht gezogen, da die Bakterien hohe Zuckerkonzentrationen tolerieren, extrazelluläre Genusssäuren bilden können und andere Essigsäurebakterien bereits in der Getränkeindustrie eingesetzt werden. Ein daraus resultierendes Getränk könnte als kalorienreduziert und natürlich vermarktet werden. Um die

grundsätzliche Eignung zu testen, wurde das Wachstum der verfügbaren Stämme in vier kommerziellen Fruchtsäften untersucht. Alle Stämme wuchsen in allen Fruchtsäften trotz hoher Zuckerkonzentrationen, niedriger pH-Werte und pflanzlicher Polyphenole mit antimikrobiellen Eigenschaften. Im nächsten Schritt wurde untersucht, ob *B. favorum* TMW 2.1880 durch UV-Mutagenese an das Fruchtsaftmilieu angepasst werden kann. Durch die Anpassung an eine hohe Glukosekonzentration, d.h. an einen einzelnen Stressor, konnte ein Stamm mit verbesserten Wachstumseigenschaften in Traubensaftkonzentrat erhalten werden. Mit Hilfe von Proteomik-Experimenten wurde untersucht, welche zellulären Funktionen durch die Mutagenese beeinflusst wurden. Es wurde festgestellt, dass das verbesserte Wachstum wahrscheinlich nicht auf eine Anpassung an hohe Glukosekonzentrationen zurückzuführen ist, sondern auf den Verlust von Flagellen, also Zellmotilität. Die hierdurch eingesparte Energie kann wohl in anderen Wachstumsprozessen genutzt werden. Die Zellen scheinen also ihren Energiehaushalt an die Kultivierung im Schüttelkolben angepasst zu haben.

Essigsäurebakterien werden auch wegen ihrer Fähigkeit, extrazelluläre Polysaccharide (EPS) zu bilden, in der Industrie eingesetzt. Da es bisher keine Untersuchungen über die EPS-Bildung durch *Bombella* spp. gegeben hat, wurde die EPS-Bildung der verfügbaren Stämme auf Sachharose-Agarplatten untersucht. Von den 12 Stämmen aus 8 verschiedenen Bombella-Spezies bildeten nur *B. apis* TMW 2.1884 und *B. mellum* TMW 2.1889 EPS. Beide EPS wurden durch AF4-MALLS/UV-Analysen als hochmolekulare Polymere (106-107 Da) identifiziert. Die extern durchgeführte Monosaccharidanalyse mittels TFA-Hydrolyse zeigte, dass beide EPS Fruktane sind. Die ebenfalls extern durchgeführte Bestimmung der glykosidischen Bindungen durch Methylierungsanalyse ergab hauptsächlich 2,6-verknüpfte Fructofuranose-Einheiten (*Fru*f) mit zusätzlichen 1,2-verknüpften *Fru*f-Einheiten (10 %) und verzweigten 1,2,6-Fruf-Einheiten (7 %). In den entsprechenden Genomen konnten keine Gene von Enzymen der „Glycoside Hydrolase Family (GH) 68“ identifiziert werden, die typischerweise mit der Bildung von hochmolekularen Fruktanen in Bakterien in Verbindung gebracht werden. Durch heterologe Expression in *E. coli* Top10 konnte ein Enzym der GH 32-Familie mit der Katalyse der Fruktanbildung in Verbindung gebracht werden. Die identifizierten Fructosyltransferasen konnten phylogenetisch und strukturell eindeutig von anderen bisher beschriebenen bakteriellen Fructosyltransferasen unterschieden werden. Diese Ergebnisse stellen die erste Beschreibung von bakteriellen Enzymen der GH 32-Familie dar, die an der Bildung von hochmolekularen Fruktanen beteiligt sind.

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8 Supplementary Data

Supplementary Table S1: Locus tags of predicted central carbohydrate metabolism and membrane-bound dehydrogenases in *Bombella* spp.. Numbers (Nr.) refer to Figure 6. Fields marked in red indicate missing genes.

Nr.	Enzyme	*Bombella apis* MRM1^T	*Bombella apis* ESL0368	*Bombella apis* ESL0380	*Bombella apis* ESL0387
1	glucokinase	IGM82_02550	GN304_04590	GM608_04215	GM609_04355
2	glucose-6-phosphate isomerase	IGM82_00320	GN304_02620	GM608_02230	GM609_02420
3	fructose-1,6-bisphosphatase	IGM82_06235	GN304_00945	GM608_00555	GM609_00550
4	fructose-bisphosphate aldolase	IGM82_08730	GN304_08075	GM608_07690	GM609_08095
5	glyceraldehyde-3-phosphate DH	IGM82_04595	GN304_07405	GM608_07045	GM609_07450
6	phosphoglycerate kinase	IGM82_04600	GN304_07400	GM608_07040	GM609_07445
7	phosphoglycerate mutase	IGM82_08185	GN304_08620	GM608_08230	GM609_08635
8	enolase	IGM82_01685	GN304_04010	GM608_03655	GM609_03820
9	triose-phosphate isomerase	IGM82_01710	GN304_04035	GM608_03680	GM609_03845
10	pyruvate kinase	IGM82_01455	GN304_03780	GM608_03425	GM609_03590
11	pyruvate dehydrogenase complex	IGM82_01740	GN304_04065	GM608_03710	GM609_03875
		IGM82_01745	GN304_04070	GM608_03715	GM609_03880
		IGM82_01750	GN304_04075	GM608_03720	GM609_03885
		IGM82_01755	GN304_04080	GM608_03725	GM609_03890
12	citrate synthase	IGM82_00420	GN304_02725	GM608_02330	GM609_02520
13	aconitate hydratase	IGM82_00425	GN304_02730	GM608_02335	GM609_02525
14	isocitrate dehydrogenase	IGM82_00415	GN304_02720	GM608_02325	GM609_02515
15	oxoglutarate dehydrogenase complex	IGM82_06575	GN304_01285	GM608_00895	GM609_00890
		IGM82_07885	GN304_00030	GM608_08530	GM609_08935
		IGM82_01755	GN304_04080	GM608_03725	GM609_03890
16	succinyl-CoA synthetase				
17	succinate dehydrogenase				
18	fumarase	IGM82_00050	GN304_02350	GM608_01950	GM609_02150
19	malate dehydrogenase				
20	pyruvate decarboxylase	IGM82_06545	GN304_01255	GM608_00865	GM609_00860
21	malate dehydrogenase (OA-decarbox)	IGM82_00055	GN304_02355	GM608_01955	GM609_02155
23	fructokinase	IGM82_07785	GN304_00130	GM608_08630	GM609_09035
24	glucose-6-phosphate dehydrogenase	IGM82_03785	GN304_05640	GM608_05270	GM609_05410
25	6-phosphogluconolactonase	IGM82_00335	GN304_02635	GM608_02245	GM609_02435
26	glucose 1-dehydrogenase	IGM82_02830	GN304_04870	GM608_04495	GM609_04635
27	gluconolactonase	IGM82_00395	GN304_02700	GM608_02305	GM609_02495
28	gluconoate kinase	IGM82_00345	GN304_02645	GM608_02255	GM609_02445
29	phosphogluconate dehydrogenase	IGM82_00325	GN304_02625	GM608_02235	GM609_02425
30	ribulose-phosphate 3-epimerase	IGM82_09070	GN304_00535	GM608_00180	GM609_00185
31	ribose-5-phosphate isomerase	IGM82_00340	GN304_02640	GM608_02250	GM609_02440
32	transketolase	IGM82_00315	GN304_02615	GM608_02225	GM609_02415
33	transaldolase	IGM82_00320	GN304_02620	GM608_02230	GM609_02420
34	6-phosphogluconate dehydratase	IGM82_05135	GN304_06870	GM608_06505	GM609_06910
35	2-dehydro-3-deoxyphosphogluconate aldolase	IGM82_05140	GN304_06865	GM608_06500	GM609_06905
	MMB DHs and respiratory enzymes				
I	PQQ-dependent glucose dehydrogenase	IGM82_05820	GN304_06185	GM608_05815	GM609_05915
II	gluconate-2 dehydrogenase	IGM82_08595	GN304_08210	GM608_07825	GM609_08230
		IGM82_08600	GN304_08205	GM608_07820	GM609_08225
		IGM82_08605	GN304_08200	GM608_07815	GM609_08220
III	quinone-dependent dihydroorotate dehydrogenase	IGM82_04325	GN304_07670	GM608_07310	GM609_07715
IV	D-lactate dehydrogenase	IGM82_08680	GN304_08125	GM608_07740	GM609_08145
V	alcohol dehydrogenase	IGM82_02895	GN304_04935	GM608_04560	GM609_04700
		IGM82_02900	GN304_04940	GM608_04565	GM609_04705
VI	alcohol dehydrogenase (copy)	IGM82_02905	GN304_04945	GM608_04570	GM609_04710
		IGM82_02910	GN304_04950	GM608_04575	GM609_04715
VII	NADH dehydrogenase (type II)	IGM82_00200	GN304_02500	GM608_02110	GM609_02300
VIII	bo3-type cytochrome c oxidase	IGM82_01325	GN304_03635	GM608_03235	GM609_03460
		IGM82_01320	GN304_03630	GM608_03230	GM609_03455

		IGM82_01330	GN304_03640	GM608_03240	GM609_03465
		IGM82_01335	GN304_03645	GM608_03245	GM609_03470
IX	cytochrome bc1 complex	IGM82_06755	GN304_01455	GM608_01065	GM609_01060
		IGM82_06760	GN304_01460	GM608_01070	GM609_01065
		IGM82_06765	GN304_01465	GM608_01075	GM609_01070
X	flavoprotein-ubiquinone oxidoreductase	IGM82_06605	GN304_01315	GM608_00925	GM609_00920
		IGM82_06610	GN304_01320	GM608_00930	GM609_00925
XI	cytochrome D ubiquinol oxidase	IGM82_07910	GN304_00005	GM608_08505	GM609_08910
		IGM82_07905	GN304_00010	GM608_08510	GM609_08915

Table S1 continued

Nr.	*Bombella apis* SME1	*Bombella apis* TMW 2.1882	*Bombella apis* TMW 2.1884	*Bombella apis* TMW 2.1886	*Bombella apis* TMW 2.1888	*Bombella apis* TMW 2.1890
1	GDY84_07150	CO583_01395	DTI93_02780	DTJ06_04320	DTQ13_09110	CPA54_03015
2	GDY84_09345	CO583_03670	DTI93_00795	DTJ06_01640	DTQ13_06895	CPA54_01045
3	GDY84_00605	CO583_05315	DTI93_05770	DTJ06_06885	DTQ13_05225	CPA54_08575
4	GDY84_02120	CO583_08655	DTI93_05360	DTJ06_07300	DTQ13_03450	CPA54_07805
5	GDY84_03665	CO583_07045	DTI93_07320	DTJ06_02455	DTQ13_02785	CPA54_06180
6	GDY84_03670	CO583_07040	DTI93_07315	DTJ06_02460	DTQ13_02780	CPA54_06175
7	GDY84_02660	CO583_08115	DTI93_04815	DTJ06_07840	DTQ13_03990	CPA54_07265
8	GDY84_07975	CO583_02270	DTI93_02195	DTJ06_00245	DTQ13_08265	CPA54_02420
9	GDY84_07950	CO583_02245	DTI93_02220	DTJ06_00220	DTQ13_08290	CPA54_02445
10	GDY84_08205	CO583_02515	DTI93_01965	DTJ06_00475	DTQ13_08035	CPA54_02190
11	GDY84_07920	CO583_02215	DTI93_02250	DTJ06_00190	DTQ13_08320	CPA54_02475
	GDY84_07915	CO583_02210	DTI93_02255	DTJ06_00185	DTQ13_08325	CPA54_02480
	GDY84_07910	CO583_02205	DTI93_02260	DTJ06_00180	DTQ13_08330	CPA54_02485
	GDY84_07905	CO583_02200	DTI93_02265	DTJ06_00175	DTQ13_08335	CPA54_02490
12	GDY84_09250	CO583_03570	DTI93_00895	DTJ06_01545	DTQ13_06990	CPA54_01145
13	GDY84_09245	CO583_03565	DTI93_00900	DTJ06_01540	DTQ13_06995	CPA54_01150
14	GDY84_09255	CO583_03575	DTI93_00890	DTJ06_01550	DTQ13_06985	CPA54_01140
15	GDY84_00945	CO583_04975	DTI93_06110	DTJ06_06525	DTQ13_05575	CPA54_08235
	GDY84_02965	CO583_07815	DTI93_04505	DTJ06_08145	DTQ13_04300	CPA54_06955
	GDY84_07905	CO583_02200	DTI93_02265	DTJ06_00175	DTQ13_08335	CPA54_02490
16						
17						
18	GDY84_09615	CO583_03945	DTI93_00525	DTJ06_01915	DTQ13_06625	CPA54_00775
19						
20	GDY84_00915	CO583_05005	DTI93_06080	DTJ06_06555	DTQ13_05540	CPA54_08265
21	GDY84_09610	CO583_03940	DTI93_00530	DTJ06_01910	DTQ13_06630	CPA54_00780
23	GDY84_03065	CO583_07720	DTI93_04405	DTJ06_08245	DTQ13_04410	CPA54_06855
24	GDY84_06100	CO583_00345	DTI93_03830	DTJ06_05370	DTQ13_00990	CPA54_04065
25	GDY84_09330	CO583_03655	DTI93_00810	DTJ06_01625	DTQ13_06910	CPA54_01060
26	GDY84_06870	CO583_01115	DTI93_03060	DTJ06_04600	DTQ13_00220	CPA54_03295
27	GDY84_09270	CO583_03595	DTI93_00870	DTJ06_01565	DTQ13_06970	CPA54_01120
28	GDY84_09320	CO583_03645	DTI93_00820	DTJ06_01615	DTQ13_06920	CPA54_01070
29	GDY84_09340	CO583_03665	DTI93_00800	DTJ06_01635	DTQ13_06900	CPA54_01050
30	GDY84_05520	CO583_08970	DTI93_08970	DTJ06_08585	DTQ13_04825	CPA54_08950
31	GDY84_09325	CO583_03650	DTI93_00815	DTJ06_01620	DTQ13_06915	CPA54_01065
32	GDY84_09350	CO583_03675	DTI93_00790	DTJ06_01645	DTQ13_06890	CPA54_01040
33	GDY84_09345	CO583_03670	DTI93_00795	DTJ06_01640	DTQ13_06895	CPA54_01045
34	GDY84_04200	CO583_06510	DTI93_06785	DTJ06_02990	DTQ13_02250	CPA54_05650
35	GDY84_04205	CO583_06505	DTI93_06780	DTJ06_02995	DTQ13_02245	CPA54_05645
I	GDY84_05220	CO583_05780	DTI93_07955	DTJ06_03690	DTQ13_01555	CPA54_04965
II	GDY84_02255	CO583_08520	DTI93_05225	DTJ06_07435	DTQ13_03585	CPA54_07670
	GDY84_02250	CO583_08525	DTI93_05230	DTJ06_07430	DTQ13_03580	CPA54_07675
	GDY84_02245	CO583_08530	DTI93_05235	DTJ06_07425	DTQ13_03575	CPA54_07680
III	GDY84_03405	CO583_07305	DTI93_07580	DTJ06_02180	DTQ13_03045	CPA54_06440
IV	GDY84_02170	CO583_08605	DTI93_05310	DTJ06_07350	DTQ13_03500	CPA54_07755
V	GDY84_06795	CO583_01040	DTI93_03125	DTJ06_04665	DTQ13_00285	CPA54_03360
	GDY84_06790	CO583_01035	DTI93_03130	DTJ06_04670	DTQ13_00290	CPA54_03365
VI	GDY84_06805	CO583_01050	DTI93_03135	DTJ06_04675	DTQ13_00295	CPA54_03370
	GDY84_06800	CO583_01045	DTI93_03140	DTJ06_04680	DTQ13_00300	CPA54_03375
VII	GDY84_09465	CO583_03790	DTI93_00675	DTJ06_01760	DTQ13_06775	CPA54_00925
VIII	GDY84_08340	CO583_02665	DTI93_01810	DTJ06_00635	DTQ13_07900	CPA54_02060
	GDY84_08345	CO583_02670	DTI93_01805	DTJ06_00640	DTQ13_07895	CPA54_02055
	GDY84_08335	CO583_02660	DTI93_01815	DTJ06_00630	DTQ13_07905	CPA54_02065
	GDY84_08330	CO583_02655	DTI93_01820	DTJ06_00625	DTQ13_07910	CPA54_02070
IX	GDY84_01115	CO583_04805	DTI93_06280	DTJ06_06355	DTQ13_05750	CPA54_08065
	GDY84_01120	CO583_04800	DTI93_06285	DTJ06_06350	DTQ13_05755	CPA54_08060
	GDY84_01125	CO583_04795	DTI93_06290	DTJ06_06345	DTQ13_05760	CPA54_08055
X	GDY84_00975	CO583_04945	DTI93_06140	DTJ06_06495	DTQ13_05605	CPA54_08205
	GDY84_00980	CO583_04940	DTI93_06145	DTJ06_06490	DTQ13_05610	CPA54_08200
XI	GDY84_02935	CO583_07840	DTI93_04535	DTJ06_08115	DTQ13_04270	CPA54_06985
	GDY84_02940	CO583_07835	DTI93_04530	DTJ06_08120	DTQ13_04275	CPA54_06980

Table S1 continued

Nr.	*Bombella apis* TMW 2.1891	*Bombella intestini* R52487^T	*Bombella favorum* TMW 2.1880^T	*Bombella mellum* TMW 2.1889^T	*Bombella apis* A29	*Bombella apis* AM169
1	DTJ15_04050	AL01_RS01630	CPA57_01510	CPA56_03355	ASO19_RS05025	SACS_RS05320
2	DTJ15_01895	AL01_RS05915	CPA57_03480	CPA56_01065	ASO19_RS03010	SACS_RS07275
3	DTJ15_00240	AL01_RS08450	CPA57_05125	CPA56_08110	ASO19_RS07605	SACS_RS08140
4	DTJ15_08270	AL01_RS06675	CPA57_07175	CPA56_06705	ASO19_RS06210	SACS_RS02475
5	DTJ15_05935	AL01_RS03630	CPA57_06525	CPA56_05350	ASO19_RS01875	SACS_RS01250
6	DTJ15_05940	AL01_RS03635	CPA57_06520	CPA56_05355	ASO19_RS01870	SACS_RS01255
7	DTJ15_07730	AL01_RS07230	CPA57_07710	CPA56_07270	ASO19_RS06755	SACS_RS03010
8	DTJ15_03265	AL01_RS00830	CPA57_02130	CPA56_02440	ASO19_RS04405	SACS_RS05930
9	DTJ15_03290	AL01_RS00855	CPA57_02105	CPA56_02465	ASO19_RS04430	SACS_RS05905
10	DTJ15_03035	AL01_RS00600	CPA57_02360	CPA56_02210	ASO19_RS04160	SACS_RS06160
11	DTJ15_03320	AL01_RS00885	CPA57_02075	CPA56_02495	ASO19_RS04460	SACS_RS05875
	DTJ15_03325	AL01_RS00890	CPA57_02070	CPA56_02500	ASO19_RS04465	SACS_RS05870
	DTJ15_03330	AL01_RS00895	CPA57_02065	CPA56_02505	ASO19_RS04470	SACS_RS05865
	DTJ15_03335	AL01_RS00900	CPA57_02060	CPA56_02510	ASO19_RS04475	SACS_RS05860
12	DTJ15_01995	AL01_RS06055	CPA57_03370		ASO19_RS03105	SACS_RS07180
13	DTJ15_02000	AL01_RS06060	CPA57_03365		ASO19_RS03110	SACS_RS07175
14	DTJ15_01990	AL01_RS06050	CPA57_03375		ASO19_RS03100	SACS_RS07185
15	DTJ15_00580	AL01_RS08105	CPA57_04770	CPA56_08475	ASO19_RS07945	SACS_RS02160
	DTJ15_07425	AL01_RS07525	CPA57_08020	CPA56_07605	ASO19_RS07060	SACS_RS03310
	DTJ15_03335	AL01_RS00900	CPA57_02060	CPA56_02510	ASO19_RS04475	SACS_RS05860
16						
17						
18	DTJ15_01625	AL01_RS05650	CPA57_03750	CPA56_00780	ASO19_RS02845	SACS_RS07550
19						
20	DTJ15_00550	AL01_RS08135	CPA57_04800	CPA56_08440	ASO19_RS07915	SACS_RS02195
21	DTJ15_01630	AL01_RS05655	CPA57_03745	CPA56_00785	ASO19_RS02840	SACS_RS07545
23	DTJ15_07330	AL01_RS07630	CPA57_08125	CPA56_07705	ASO19_RS07160	SACS_RS03410
24	DTJ15_05100	AL01_RS02715	CPA57_00440	CPA56_04440	ASO19_RS00095	SACS_RS04275
25	DTJ15_01910	AL01_RS05930	CPA57_03465	CPA56_01080	ASO19_RS03025	SACS_RS07260
26	DTJ15_04330	AL01_RS01945	CPA57_01205	CPA56_03640	ASO19_RS05305	SACS_RS05040
27	DTJ15_01970	AL01_RS06030	CPA57_03395	CPA56_01140	ASO19_RS03085	SACS_RS07200
28	DTJ15_01920	AL01_RS05940	CPA57_03455	CPA56_01090	ASO19_RS03035	SACS_RS07250
29	DTJ15_01900	AL01_RS05920	CPA57_03475	CPA56_01070	ASO19_RS03015	SACS_RS07270
30	DTJ15_08585	AL01_RS08800	CPA57_08505	CPA56_08875	ASO19_RS02500	SACS_RS03760
31	DTJ15_01915	AL01_RS05935	CPA57_03460	CPA56_01085	ASO19_RS03030	SACS_RS07255
32	DTJ15_01890	AL01_RS05910	CPA57_03485	CPA56_01060	ASO19_RS03005	SACS_RS07280
33	DTJ15_01895	AL01_RS05915	CPA57_03480	CPA56_01065	ASO19_RS03010	SACS_RS07275
34	DTJ15_06480	AL01_RS04185	CPA57_05955	CPA56_05885	ASO19_RS01330	SACS_RS01785
35	DTJ15_06485	AL01_RS04190	CPA57_05950	CPA56_05890	ASO19_RS01325	SACS_RS01790
I	DTJ15_08930	AL01_RS09040	CPA57_08725	CPA56_09190	ASO19_RS00605	SACS_RS00600
II	DTJ15_08135	AL01_RS06810	CPA57_07310	CPA56_06850	ASO19_RS06345	SACS_RS02605
	DTJ15_08140	AL01_RS06805	CPA57_07305	CPA56_06845	ASO19_RS06340	SACS_RS02600
	DTJ15_08145	AL01_RS06800	CPA57_07300	CPA56_06840	ASO19_RS06335	SACS_RS02595
III	DTJ15_05670	AL01_RS03365	CPA57_06790	CPA56_05085	ASO19_RS02150	SACS_RS00990
IV	DTJ15_08220	AL01_RS06725	CPA57_07225	CPA56_06755	ASO19_RS06260	SACS_RS02520
V	DTJ15_04395	AL01_RS02010	CPA57_01130	CPA56_03705	ASO19_RS05370	SACS_RS04965
	DTJ15_04400	AL01_RS02015	CPA57_01125	CPA56_03710	ASO19_RS05375	SACS_RS04960
VI	DTJ15_04405	AL01_RS02020	CPA57_01140	CPA56_03715	ASO19_RS05380	SACS_RS04975
	DTJ15_04410	AL01_RS02025	CPA57_01135	CPA56_03720	ASO19_RS05385	SACS_RS04970
VII	DTJ15_01775	AL01_RS05790	CPA57_03600	CPA56_00940	ASO19_RS02690	SACS_RS07395
VIII	DTJ15_02905	AL01_RS00455	CPA57_02485	CPA56_02080	ASO19_RS04030	SACS_RS06290
	DTJ15_02900	AL01_RS00450	CPA57_02490	CPA56_02075	ASO19_RS04025	SACS_RS06295
	DTJ15_02910	AL01_RS00460	CPA57_02480	CPA56_02085	ASO19_RS04035	SACS_RS06285
	DTJ15_02915	AL01_RS00465	CPA57_02475	CPA56_02090	ASO19_RS04040	SACS_RS06280
IX	DTJ15_00750	AL01_RS07925	CPA57_04595	CPA56_08650	ASO19_RS08115	SACS_RS01985
	DTJ15_00755	AL01_RS07920	CPA57_04590	CPA56_08655	ASO19_RS08120	SACS_RS01980
	DTJ15_00760	AL01_RS07915	CPA57_04585	CPA56_08660	ASO19_RS08125	SACS_RS01975
X	DTJ15_00610	AL01_RS08075	CPA57_04740	CPA56_08505	ASO19_RS07975	SACS_RS02130
	DTJ15_00615	AL01_RS08070	CPA57_04735	CPA56_08510	ASO19_RS07980	SACS_RS02125
XI	DTJ15_07455		CPA57_07990	CPA56_07570	ASO19_RS07035	SACS_RS03285
	DTJ15_07450		CPA57_07995	CPA56_07575	ASO19_RS07040	SACS_RS03290

Table S1 continued

Nr.	*Bombella apis* G773c	*Bombella apis* 3A1	*Bombella apis* M18	*B. pluederhaus.* TMW 2.2543^{T}	*Bombella pollinis* TMW 2.2556^{T}	*Bombella pollinis* ESL0378
1	B6V90_RS07400	BLA18_RS04130	BK793_RS04005	NQF86_03190	NQF89_08440	GM556_RS03795
2	B6V90_RS05420	BLA18_RS01660	BK793_RS01715	NQF86_01050	NQF89_04105	GM556_RS01955
3	B6V90_RS01565	BLA18_RS07120	BK793_RS03390	NQF86_08345	NQF89_00910	GM556_RS00325
4	B6V90_RS04910	BLA18_RS05745	BK793_RS06605	NQF86_07555	NQF89_07910	GM556_RS07205
5	B6V90_RS03715	BLA18_RS03400	BK793_RS08230	NQF86_05115	NQF89_06200	GM556_RS06560
6	B6V90_RS03710	BLA18_RS03395	BK793_RS08225	NQF86_05120	NQF89_06195	GM556_RS06555
7	B6V90_RS00355	BLA18_RS06285	BK793_RS06065	NQF86_07005	NQF89_01430	GM556_RS07870
8	B6V90_RS06855	BLA18_RS00225	BK793_RS00335	NQF86_02500	NQF89_05455	GM556_RS03305
9	B6V90_RS06880	BLA18_RS00200	BK793_RS00310	NQF86_02525	NQF89_05480	GM556_RS03330
10	B6V90_RS06625	BLA18_RS00455	BK793_RS00565	NQF86_02270	NQF89_05225	GM556_RS03080
11	B6V90_RS06910	BLA18_RS00170	BK793_RS00280	NQF86_02555	NQF89_05510	GM556_RS03360
	B6V90_RS06915	BLA18_RS00165	BK793_RS00275	NQF86_02560	NQF89_05515	GM556_RS03365
	B6V90_RS06920	BLA18_RS00160	BK793_RS00270	NQF86_02565	NQF89_05520	GM556_RS03370
	B6V90_RS06925	BLA18_RS00155	BK793_RS00265	NQF86_02570	NQF89_05525	GM556_RS03375
12	B6V90_RS05515	BLA18_RS01560	BK793_RS01615	NQF86_01190		
13	B6V90_RS05520	BLA18_RS01555	BK793_RS01610	NQF86_01195		
14	B6V90_RS05510	BLA18_RS01565	BK793_RS01620	NQF86_01185		
15	B6V90_RS01230	BLA18_RS07460	BK793_RS03045	NQF86_07990	NQF89_00565	GM556_RS00665
	B6V90_RS00660	BLA18_RS06585	BK793_RS05765	NQF86_06695	NQF89_01690	GM556_RS08125
	B6V90_RS06925	BLA18_RS00155	BK793_RS00265	NQF86_02570	NQF89_05525	GM556_RS03375
16						
17						
18	B6V90_RS05150	BLA18_RS01935	BK793_RS01990	NQF86_00780	NQF89_03850	GM556_RS01705
19						
20	B6V90_RS01260	BLA18_RS07430	BK793_RS03075	NQF86_08020	NQF89_00595	GM556_RS00635
21	B6V90_RS05155	BLA18_RS01930	BK793_RS01985	NQF86_00785	NQF89_03855	GM556_RS01710
23	B6V90_RS00760	BLA18_RS06685	BK793_RS05670	NQF86_06580	NQF89_01790	GM556_RS08225
24	B6V90_RS08450	BLA18_RS05180	BK793_RS05065	NQF86_04280	NQF89_03025	GM556_RS04835
25	B6V90_RS05435	BLA18_RS01645	BK793_RS01700	NQF86_01065	NQF89_04120	GM556_RS01970
26	B6V90_RS07680	BLA18_RS04410	BK793_RS04285	NQF86_03485	NQF89_02275	GM556_RS04090
27	B6V90_RS05495	BLA18_RS01585	BK793_RS01640	NQF86_01165	NQF89_04180	GM556_RS02030
28	B6V90_RS05445	BLA18_RS01635	BK793_RS01690	NQF86_01075	NQF89_04130	GM556_RS01980
29	B6V90_RS05425	BLA18_RS01655	BK793_RS01710	NQF86_01055	NQF89_04110	GM556_RS01960
30	B6V90_RS01955	BLA18_RS08635	BK793_RS08895	NQF86_08625	NQF89_08215	GM556_RS00180
31	B6V90_RS05440	BLA18_RS01640	BK793_RS01695	NQF86_01070	NQF89_04125	GM556_RS01975
32	B6V90_RS05415	BLA18_RS01665	BK793_RS01720	NQF86_01045	NQF89_04100	GM556_RS01950
33	B6V90_RS05420	BLA18_RS01660	BK793_RS01715	NQF86_01050	NQF89_04105	GM556_RS01955
34	B6V90_RS02960	BLA18_RS02865	BK793_RS07685	NQF86_05700	NQF89_06840	GM556_RS06035
35	B6V90_RS02955	BLA18_RS02860	BK793_RS07680	NQF86_05705	NQF89_06845	GM556_RS06030
I	B6V90_RS08885	BLA18_RS02150	BK793_RS06945	NQF86_08960	NQF89_07510	GM556_RS05360
II	B6V90_RS05045	BLA18_RS05880	BK793_RS06470	NQF86_07420	NQF89_07775	GM556_RS07340
	B6V90_RS05040	BLA18_RS05875	BK793_RS06475	NQF86_07425	NQF89_07780	GM556_RS07335
	B6V90_RS05035	BLA18_RS05870	BK793_RS06480	NQF86_07430	NQF89_07785	GM556_RS07330
III	B6V90_RS03980	BLA18_RS03660	BK793_RS08490	NQF86_04845	NQF89_06470	GM556_RS06825
IV	B6V90_RS04960	BLA18_RS05795	BK793_RS06555	NQF86_07505	NQF89_07860	GM556_RS07255
V	B6V90_RS07745	BLA18_RS04475	BK793_RS04350	NQF86_03550	NQF89_02340	GM556_RS04155
	B6V90_RS07750	BLA18_RS04480	BK793_RS04355	NQF86_03555	NQF89_02345	GM556_RS04160
VI	B6V90_RS07755	BLA18_RS04485	BK793_RS04360	NQF86_03560		
	B6V90_RS07760	BLA18_RS04490	BK793_RS04365	NQF86_03565		
VII	B6V90_RS05300	BLA18_RS01780	BK793_RS01835	NQF86_00930	NQF89_03985	GM556_RS01835
VIII	B6V90_RS06445	BLA18_RS00650	BK793_RS00705	NQF86_02110	NQF89_05090	GM556_RS02940
	B6V90_RS06440	BLA18_RS00655	BK793_RS00710	NQF86_02105	NQF89_05085	GM556_RS02935
	B6V90_RS06450	BLA18_RS00645	BK793_RS00700	NQF86_02115	NQF89_05095	GM556_RS02945
	B6V90_RS06455	BLA18_RS00640	BK793_RS00695	NQF86_02120	NQF89_05100	GM556_RS02950
IX	B6V90_RS01060	BLA18_RS07635	BK793_RS02875	NQF86_07825	NQF89_00400	GM556_RS00825
	B6V90_RS01055	BLA18_RS07640	BK793_RS02870	NQF86_07820	NQF89_00395	GM556_RS00830
	B6V90_RS01050	BLA18_RS07645	BK793_RS02865	NQF86_07815	NQF89_00390	GM556_RS00835
X	B6V90_RS01200	BLA18_RS07490	BK793_RS03015	NQF86_07960	NQF89_00535	GM556_RS00695
	B6V90_RS01195	BLA18_RS07495	BK793_RS03010	NQF86_07955	NQF89_00530	GM556_RS00700
XI	B6V90_RS00635	BLA18_RS06560	BK793_RS05790	NQF86_06725	NQF89_01665	GM556_RS08100
	B6V90_RS00640	BLA18_RS06565	BK793_RS05785	NQF86_06720	NQF89_01670	GM556_RS08105

Table S1 continued

Nr.	*Bombella pollinis* ESL0385	*B. saccharophila* TMW 2.2558^T	*B.* saccharophila AS1	*Bombella dulcis* TMW 2.2559^T
1	GM557_RS04785	NQF64_02635	BG621_RS07340	NQF87_02000
2	GM557_RS02715	NQF64_00730	BG621_RS01500	NQF87_05030
3	GM557_RS01085	NQF64_05300	BG621_RS03010	NQF87_03570
4	GM557_RS08275	NQF64_08450	BG621_RS07840	NQF87_06285
5	GM557_RS07625	NQF64_06570	BG621_RS05440	NQF87_07910
6	GM557_RS07620	NQF64_06575	BG621_RS05435	NQF87_07915
7	GM557_RS00570	NQF64_04805	BG621_RS03525	NQF87_05760
8	GM557_RS04065	NQF64_02045	BG621_RS00200	NQF87_01465
9	GM557_RS04090	NQF64_02070	BG621_RS00175	NQF87_01490
10	GM557_RS03840	NQF64_01835	BG621_RS00410	NQF87_01230
11	GM557_RS04120	NQF64_02100	BG621_RS00145	NQF87_01520
	GM557_RS04125	NQF64_02105	BG621_RS00140	NQF87_01525
	GM557_RS04130	NQF64_02110	BG621_RS00135	NQF87_01530
	GM557_RS04135	NQF64_02115	BG621_RS00130	NQF87_01535
12				NQF87_00145
13				NQF87_00150
14				NQF87_00140
15	GM557_RS01430	NQF64_05640	BG621_RS02665	NQF87_03915
	GM557_RS00305	NQF64_04540	BG621_RS03785	NQF87_05470
	GM557_RS04135	NQF64_02115	BG621_RS00130	NQF87_01535
16				
17				
18	GM557_RS02465	NQF64_00475	BG621_RS01750	NQF87_04750
19				
20	GM557_RS01395	NQF64_05610	BG621_RS02695	NQF87_03880
21	GM557_RS02470	NQF64_00480	BG621_RS01745	NQF87_04755
23	GM557_RS00205	NQF64_04440	BG621_RS03885	NQF87_05370
24	GM557_RS05835	NQF64_03690	BG621_RS06580	NQF87_03060
25	GM557_RS02730	NQF64_00745	BG621_RS01485	NQF87_05045
26	GM557_RS05090	NQF64_02940	BG621_RS07045	NQF87_02285
27	GM557_RS02790	NQF64_00805	BG621_RS01425	NQF87_00120
28	GM557_RS02740	NQF64_00755	BG621_RS01475	NQF87_05055
29	GM557_RS02720	NQF64_00735	BG621_RS01495	NQF87_05035
30	GM557_RS08645	NQF64_08065	BG621_RS08115	NQF87_08170
31	GM557_RS02735	NQF64_00750	BG621_RS01480	NQF87_05050
32	GM557_RS02710	NQF64_00725	BG621_RS01505	NQF87_05025
33	GM557_RS02715	NQF64_00730	BG621_RS01500	NQF87_05030
34	GM557_RS07100	NQF64_07100	BG621_RS04920	NQF87_07315
35	GM557_RS07095	NQF64_07105	BG621_RS04915	NQF87_07310
I	GM557_RS06430	NQF64_07775	BG621_RS04245	NQF87_06625
II	GM557_RS08410	NQF64_08585	BG621_RS07975	NQF87_06150
	GM557_RS08405	NQF64_08580	BG621_RS07970	NQF87_06155
	GM557_RS08400	NQF64_08575	BG621_RS07965	NQF87_06160
III	GM557_RS07890	NQF64_06300	BG621_RS05705	NQF87_07635
IV	GM557_RS08325	NQF64_08500	BG621_RS07890	NQF87_06235
V	GM557_RS05155	NQF64_03005	BG621_RS06975	NQF87_02350
	GM557_RS05160	NQF64_03010	BG621_RS06980	NQF87_02355
VI				
		NQF64_03015		
VII	GM557_RS02595	NQF64_00610	BG621_RS01620	NQF87_04900
VIII	GM557_RS03705	NQF64_01700	BG621_RS00545	NQF87_01075
	GM557_RS03700	NQF64_01695	BG621_RS00550	NQF87_01070
	GM557_RS03710	NQF64_01705	BG621_RS00540	NQF87_01080
	GM557_RS03715	NQF64_01710	BG621_RS00535	NQF87_01085
IX	GM557_RS01590	NQF64_05805	BG621_RS02505	NQF87_04085
	GM557_RS01595	NQF64_05810	BG621_RS02500	NQF87_04090
	GM557_RS01600	NQF64_05815	BG621_RS02495	NQF87_04095
X	GM557_RS01460	NQF64_05670	BG621_RS02635	NQF87_03945
	GM557_RS01465	NQF64_05675	BG621_RS02630	NQF87_03950
XI	GM557_RS00330	NQF64_04565	BG621_RS03760	NQF87_05500
	GM557_RS00325	NQF64_04560	BG621_RS03765	NQF87_05495

Supplementary Table S2: Locus tags of *Bombella* genes encoding for extracellular enzymes with potential invertase activity (GH 32 family).

Strain	**Locus Tag**
Bombella apis MRM1^{T}	IGM82_08125
Bombella apis ESL0368	GN304_08680
Bombella apis ESL0380	GM608_08290
Bombella apis ESL0387	GM609_08695
Bombella apis SME1	GDY84_02720
Bombella apis TMW 2.1882	CO583_08055
Bombella apis TMW 2.1884	DTI93_04755
Bombella apis TMW 2.1886	DTJ06_07900
Bombella apis TMW 2.1888	DTQ13_04050
Bombella apis TMW 2.1890	CPA54_07205
Bombella apis TMW 2.1891	DTJ15_07670
Bombella intestini R52487^{T}	AL01_RS07290
Bombella favorum TMW 2.1880^{T}	CPA57_07775
Bombella mellum TMW 2.1889^{T}	CPA56_07330
Bombella apis A29	ASO19_RS06815
Bombella apis AM169	SACS_RS03070
Bombella apis G773c	B6V90_RS00415
Bombella apis 3A1	BLA18_RS06345
Bombella apis M18	BK793_RS06005
Bombella pluederhausensis TMW 2.2543^{T}	NQF86_06940
Bombella pollinis TMW 2.2556^{T}	NQF89_01485
Bombella pollinis ESL0378	GM556_RS07925
Bombella pollinis ESL0385	GM557_RS00515
Bombella saccharophila TMW 2.2558^{T}	NQF64_04750
Bombella saccharophila AS1	BG621_RS03580
Bombella dulcis TMW 2.2559^{T}	NQF87_05700

Supplementary Table S3: Genome accessions for contigs of type 1 polyketide synthase gene clusters

AAB Strain	T1PKS Contig
Bombella apis MRM1^T	JADAQV0000000004
Bombella apis ESL0368	CP046394.1
Bombella apis ESL0380	WNIH01000008.1
Bombella apis ESL0387	WNIK01000007.1
Bombella apis SME1	WHNS01000005.1
Bombella apis TMW 2.1882	NWUT03
Bombella apis TMW 2.1884	QORR02
Bombella apis TMW 2.1886	QORS05
Bombella apis TMW 2.1888	CP030979
Bombella apis TMW 2.1890	NWUV03
Bombella apis TMW 2.1891	QORT03
Bombella intestini R52487^T	NZ_JATM01000004
Bombella sp. ESL0378	NZ_WNIF01000010
Bombella sp. ESL0385	NZ_WNIJ01000001
Bombella favorum TMW 2.1880^T	NWUS03
Bombella mellum TMW 2.1889^T	PDLY04
Bombella apis A29	NZ_LMYH01000013
Bombella apis AM169	NZ_CBLY010000004
Bombella sp. AS1	NZ_MEJG01000002
Bombella apis G773c	NZ_CP020554.1
Bombella apis 3A1	NZ_MNPT01000004
Bombella apis M18	NZ_MNPS01000003
Saccharibacter floricola DSM 15669	NZ_KB899335.1
Gluconacetobacter diazotrophicus PA1 5	CP001189.1
Asaia bogorensis NBRC 16594	NZ_AP014690.1

Supplementary Table S4: Intracellular metabolites of *B. favorum* TMW 2.1880 that differ significantly when cells were incubated for 5 h in glucose stress medium (400 g/L glucose) or standard medium (50 g/L glucose). Positive log10 fold change values indicate higher amounts under glucose stress, negative without stress. Metabolites with a grey background where detected in negative mode, all others in positive mode. Metabolites are statistically differentially present, if the false discovery rate is below 0.05 and the log10 fold change is higher than 0.30103 or below -0.30103.

Formula	Possible Metabolite	log10 fc	FDR
$C_4H_{10}O_4$	Erythritol	3.3924	0.0028
$C_5H_9NO_2$	Proline	2.5468	0.0016
$C_6H_{12}O_6$	Glucose	2.4928	0.0006
$C_5H_{10}O_5$	Arabinose	2.3369	0.0275
$C_6H_{12}O_6$	Glucose	2.1886	0.0015
$C_3H_6O_3$	Glyceraldehyde	2.1531	0.0084
$C_5H_{12}O_5$	Arabitol	0.6535	0.0135
$C_9H_{14}N_3O_8P$	Cytidine-5'-monophosphate	-0.3404	0.0217
$C_9H_{13}N_2O_9P$	5'UMP-diNa	-0.3822	0.0331
$C_{26}H_{52}NO_7P$	Lyso PC 18:1	-0.4321	0.0314
$C_{12}H_{10}N_2$	Harman	-0.5024	0.004
$C_{11}H_8N_2$	Beta-Carboline	-0.5206	0.0069
$C_{10}H_{12}N_4O_5$	Inosine	-0.5564	0.004
$C_{27}H_{33}N_9O_{15}P_2$	FAD	-0.5763	0.0023
$C_{23}H_{46}NO_7P$	Lyso PE 18:1	-0.6959	0.0026
$C_{23}H_{46}NO_7P$	Lyso PE 18:1	-0.7705	0.0145
$C_20H_{24}N_{10}O_{14}P_2$	Cyclic di-GMP	-0.8309	0.0023
$C_{10}H_{15}N_5O_{10}P_2$	ADP	-0.9025	0.0025
$C_8H_{16}N_2O_3$	Nalpha-Acetyl-L-lysine	-0.9494	0.0124
$C_{12}H_{10}N_4O_2$	7,8-dimethylalloxazine	-0.9744	0.0019
$C_{21}H_{27}N_7O_{14}P_2$	NAD	-1.0457	0.0025
$C_{18}H_{32}O_{16}$	Raffinose	-1.4806	0.0021
$C_{23}H_{38}N_7O_{17}P_3S$	Acetyl-CoA	-1.6554	0.0027

Supplementary Table S5: Intracellular metabolites of *B. favorum* TMW 2.1880 that differ significantly when cells were incubated for 5 h in NaCl stress medium (50 g/L NaCl) or standard medium without NaCl. Positive log10 fold change values indicate higher amounts under glucose stress, negative without stress. Metabolites with a grey background where detected in negative mode, all others in positive mode. Metabolites are statistically differentially present, if the false discovery rate is below 0.05 and the log10 fold change is higher than 0.30103 or below -0.30103.

Formula	Possible Metabolite	log10 fc	FDR
$C_4H_{10}O_4$	Erythritol	2.6293	0.0046
$C_6H_{12}O_6$	Glucose	1.8655	0.0011
$C_3H_6O_3$	Glyceraldehyde	1.4538	0.0216
$C_6H_{12}O_6$	Glucose	1.3881	0.0015
$C_5H_9NO_2$	Proline	0.9988	0.0136
$C_9H_{12}N_2O_6$	Uridine	0.8846	0.0272
$C_{10}H_{12}N_5O_7P$	Cyclic GMP	0.3149	0.012
$C_{12}H_{10}N_2$	Harman	0.3125	0.0204
$C_5H_5N_5O$	Guanine	0.3027	0.0109
$C_{10}H_{12}N_4O_5$	Inosine	-0.4084	0.0041
$C_{27}H_{33}N_9O_{15}P_2$	FAD	-0.4654	0.0052
$C_{14}H_{20}N_6O_5S$	S-Adenosyl-homocysteine	-0.4683	0.0166
$C_{11}H_{15}N_5O_3S$	5'-Methylthioadenosine	-0.576	0.0093
$C_{10}H_{14}N_5O_8P$	GMP	-0.6723	0.0006
$C_6H_{10}O_6$	Gluconolactone	-0.6748	0.0305
$C_{10}H_{17}N_3O_6S$	Glutathione	-0.75	0.0033
$C_8H_{16}N_2O_3$	Nalpha-Acetyl-L-lysine	-0.7629	0.0069
$C_{10}H_{14}N_5O_7P$	AMP	-0.7758	0.0027
$C_{10}H_{14}N_5O_7P$	AMP	-0.7869	0.0011
$C_{10}H_{15}N_5O_{10}P_2$	ADP	-1.0354	0.0018
$C_{21}H_{27}N_7O_{14}P_2$	NAD	-1.0685	0.0025
$C_9H_{14}N_3O_8P$	Cytidine-5'-monophosphate	-1.2648	0.0042
$C_{11}H_{17}NO_8$	N-Fructosyl pyroglutamate	-1.3725	0.0407
$C_{23}H_{38}N_7O_{17}P_3S$	Acetyl-CoA	-1.3975	0.0006
$C_9H_{14}N_2O_{12}P_2$	UDP-diNa	-1.5256	0.0027
$C_6H_{10}O_7$	Sorbosonic acid	-1.5489	0.0309
$C_9H_{13}N_2O_9P$	5’UMP-diNa	-1.5514	0.0033
$C_{18}H_{32}O_{16}$	Raffinose	-2.0517	0.0028

Supplementary Table S6: Significantly differentially expressed proteins between the adapted strain and the wild-type strain of *B. favorum* TMW 2.1880, when grown in glucose stress medium. Positive log10 fold change values indicate up-regulation, negative down-regulation. Proteins are statistically differentially expressed, if the false discovery rate is below 0.05 and the log10 fold change is higher than 0.30103 or below -0.30103. COG = cluster of orthologous genes; log10 fc = log10 fold change; FDR = false discovery rate.

Locus Tag	PGAP annotation	COG	log10 fc	FDR
CPA57_05865	TonB-dependent siderophore receptor	P	0.8535	0.0146
CPA57_08320	hypothetical protein	S	0.4654	0.0183
CPA57_06160	hypothetical protein		0.4273	0.0102
CPA57_05165	carbohydrate-binding protein	M	0.4037	0.0053
CPA57_06495	alkene reductase	C	0.3850	0.0039
CPA57_03390	hypothetical protein	L	0.3770	0.0043
CPA57_08015	hypothetical protein	I	0.3490	0.0109
CPA57_07365	esterase		0.3105	0.0088
CPA57_00590	integration host factor subunit alpha		-0.3011	0.0341
CPA57_06165	hypothetical protein		-0.3159	0.0074
CPA57_04850	sulfate adenylyltransferase subunit 2		-0.3417	0.0065
CPA57_06270	hypothetical protein		-0.3538	0.0066
CPA57_06380	flagellar basal body L-ring protein FlgH		-0.3669	0.0051
CPA57_06825	DNA-binding response regulator		-0.3691	0.0018
CPA57_02545	UDP-glucose 4-epimerase GalE	M	-0.3698	0.0068
CPA57_05250	sulfite reductase	C	-0.3708	0.0017
CPA57_06440	DNA-binding response regulator	T	-0.3762	0.0009
CPA57_06345	flagellar motor switch protein FliN	N	-0.3807	0.0067
CPA57_07685	transcriptional regulator	K	-0.3895	0.0109
CPA57_06330	hypothetical protein	N	-0.3916	0.0251
CPA57_00125	hypothetical protein		-0.3932	0.0087
CPA57_05245	oxidoreductase	S	-0.3986	0.0027
CPA57_06355	flagellar motor switch protein FliG	N	-0.3997	0.0011
CPA57_06430	flagellar motor switch protein FliM	N	-0.4078	0.0072
CPA57_06175	flagellar biosynthesis protein FlgB	N	-0.4170	0.0103
CPA57_05255	hypothetical protein	S	-0.4215	0.0043
CPA57_06230	hypothetical protein		-0.4290	0.0212
CPA57_06320	glycosyltransferase	M	-0.4362	0.0005
CPA57_06360	flagellar M-ring protein FliF	N	-0.4396	0.0009
CPA57_06880	hypothetical protein	S	-0.4531	0.0011
CPA57_06425	hypothetical protein		-0.4544	0.0006
CPA57_06385	flagella basal body P-ring formation protein FlgA	N	-0.4607	0.0024
CPA57_06375	hypothetical protein		-0.4743	0.0114
CPA57_06080	hypothetical protein	T	-0.4862	0.0069

CPA57_06295	hypothetical protein		-0.4931	0.0011
CPA57_06315	hypothetical protein	Q	-0.5090	0.0005
CPA57_06090	chemotaxis protein	NT	-0.5111	0.0102
CPA57_06240	hypothetical protein		-0.5121	0.0013
CPA57_06265	hypothetical protein	S	-0.5122	0.0011
CPA57_06285	glycosyl transferase	M	-0.5186	0.0005
CPA57_06085	chemotaxis response regulator protein-glutamate methylesterase	NT	-0.5233	0.0010
CPA57_06435	flagellar basal body-associated protein FliL	N	-0.5246	0.0067
CPA57_06290	hypothetical protein		-0.5306	0.0117
CPA57_06070	hypothetical protein		-0.5344	0.0027
CPA57_06180	hypothetical protein		-0.5366	0.0024
CPA57_06185	hypothetical protein		-0.5371	0.0046
CPA57_06235	UDP-galactopyranose mutase	M	-0.5380	0.0005
CPA57_06095	chemotaxis protein CheW	NT	-0.5484	0.0005
CPA57_06245	flagellar biosynthesis protein FlgD	N	-0.5734	0.0008
CPA57_06255	flagellar motor stator protein MotA	N	-0.5738	0.0244
CPA57_06105	response regulator	T	-0.5791	0.0008
CPA57_06350	hypothetical protein		-0.5823	0.0051
CPA57_06305	flagellar biosynthesis protein FlgA	N	-0.5834	0.0013
CPA57_05260	uroporphyrinogen-III C-methyltransferase	H	-0.5910	0.0013
CPA57_06100	chemotaxis protein CheA	NT	-0.6074	0.0002
CPA57_06110	hypothetical protein		-0.6104	0.0029
CPA57_06210	flagellar biosynthesis protein FlgE	N	-0.6197	0.0024
CPA57_06370	hypothetical protein	S	-0.6218	0.0011
CPA57_03425	globin-coupled sensor protein	NT	-0.6383	0.0005
CPA57_06115	chemotaxis protein	T	-0.6420	0.0001
CPA57_06190	flagellar hook-basal body complex protein FliE	N	-0.6503	0.0033
CPA57_06885	glycosyl transferase	M	-0.6836	0.0000
CPA57_06170	flagellar basal body rod protein FlgC	N	-0.7264	0.0002
CPA57_06390	flagellar basal-body rod protein FlgG	N	-0.7531	0.0011
CPA57_05230	taurine dioxygenase	Q	-0.7804	0.0002
CPA57_06325	flagellar biosynthesis protein FlhA	N	-0.8948	0.0024
CPA57_06250	hypothetical protein		-0.9201	0.0005
CPA57_06400	flagellar hook-associated protein FlgK	N	-0.9276	0.0005
CPA57_06280	hypothetical protein	S	-0.9336	0.0001
CPA57_06420	flagellin C	N	-0.9819	0.0001
CPA57_06395	flagellar basal-body rod protein FlgF	N	-1.0467	0.0043
CPA57_06275	flagellum-specific ATP synthase FliI	NU	-1.1374	0.0039
CPA57_06405	flagellar biosynthesis protein FlgL	N	-1.1767	0.0006
CPA57_04515	hypothetical protein		-1.4775	0.0099

Supplementary Table S7: Significantly differentially expressed proteins between the adapted strain and the wild-type strain of *B. favorum* TMW 2.1880, when grown in grape juice concentrate with a total sugar content of 200 g/L. Positive log10 fold change values indicate up-regulation, negative down-regulation. Proteins are statistically differentially expressed, if the false discovery rate is below 0.05 and the log10 fold change is higher than 0.30103 or below -0.30103. COG = cluster of orthologous genes; log10 fc = log10 fold change; FDR = false discovery rate.

Locus Tag	PGAP annotation	COG	log10 fc	FDR
CPA57_05315	hypothetical protein	U	1.0704	0.0087
CPA57_06215	hypothetical protein	K	1.0156	0.0086
CPA57_04630	hypothetical protein	M	0.8367	0.0084
CPA57_03835	hypothetical protein	U	0.6884	0.0277
CPA57_04505	hypothetical protein	Q	0.6214	0.0005
CPA57_05210	hypothetical protein	Q	0.6108	0.0002
CPA57_05325	hypothetical protein	S	0.5841	0.0007
CPA57_08240	hypothetical protein	Q	0.5188	0.0012
CPA57_03510	DNA-binding response regulator	KT	0.4836	0.0087
CPA57_04450	hypothetical protein	C	0.4638	0.0013
CPA57_05895	hypothetical protein	U	0.4284	0.0004
CPA57_01590	hypothetical protein		0.4177	0.0035
CPA57_03880	hypothetical protein	S	0.4043	0.0120
CPA57_07345	NADH-dependent alcohol dehydrogenase	C	0.3542	0.0017
CPA57_04785	hypothetical protein		0.3376	0.0043
CPA57_05865	TonB-dependent siderophore receptor	P	0.3368	0.0017
CPA57_00225	hypothetical protein		0.3250	0.0039
CPA57_06155	hypothetical protein		0.3012	0.0001
CPA57_07045	hypothetical protein	J	-0.3055	0.0027
CPA57_06235	UDP-galactopyranose mutase	M	-0.3129	0.0007
CPA57_06880	hypothetical protein	S	-0.3673	0.0010
CPA57_05240	heme receptor	P	-0.3703	0.0067
CPA57_06705	hypothetical protein	S	-0.3874	0.0031
CPA57_06375	hypothetical protein		-0.4160	0.0404
CPA57_01575	oxidoreductase	S	-0.4327	0.0164
CPA57_06165	hypothetical protein	S	-0.4462	0.0093
CPA57_06440	DNA-binding response regulator	T	-0.4510	0.0024
CPA57_06290	hypothetical protein		-0.4586	0.0012
CPA57_06230	hypothetical protein		-0.4758	0.0011
CPA57_06330	hypothetical protein	N	-0.4801	0.0340
CPA57_04020	hypothetical protein	M	-0.4806	0.0485
CPA57_06315	hypothetical protein	Q	-0.5025	0.0091
CPA57_06430	flagellar motor switch protein FliM	N	-0.5088	0.0005
CPA57_06425	hypothetical protein		-0.5107	0.0012

CPA57_06435	flagellar basal body-associated protein FliL	N	-0.5133	0.0017
CPA57_06320	glycosyltransferase	M	-0.5219	0.0012
CPA57_06825	DNA-binding response regulator	T	-0.5327	0.0019
CPA57_06090	chemotaxis protein	NT	-0.5350	0.0017
CPA57_06095	chemotaxis protein CheW	NT	-0.5371	0.0044
CPA57_06285	glycosyl transferase	M	-0.5426	0.0034
CPA57_06360	flagellar M-ring protein FliF	N	-0.5456	0.0005
CPA57_06355	flagellar motor switch protein FliG	N	-0.5481	0.0000
CPA57_06085	chemotaxis response regulator protein-glutamate methylesterase	NT	-0.5540	0.0001
CPA57_06080	hypothetical protein	T	-0.5611	0.0009
CPA57_06345	flagellar motor switch protein FliN	N	-0.5671	0.0027
CPA57_06105	response regulator	T	-0.5679	0.0000
CPA57_06295	hypothetical protein		-0.5702	0.0009
CPA57_06115	chemotaxis protein	T	-0.5761	0.0001
CPA57_06070	hypothetical protein		-0.5814	0.0012
CPA57_06885	glycosyl transferase	M	-0.5871	0.0001
CPA57_06280	hypothetical protein	S	-0.5890	0.0009
CPA57_06180	hypothetical protein		-0.5900	0.0005
CPA57_06100	chemotaxis protein CheA	NT	-0.5944	0.0010
CPA57_06380	flagellar basal body L-ring protein FlgH	N	-0.6026	0.0001
CPA57_06270	hypothetical protein		-0.6068	0.0025
CPA57_06370	hypothetical protein	S	-0.6154	0.0007
CPA57_06385	flagella basal body P-ring formation protein FlgA	N	-0.6177	0.0007
CPA57_06420	flagellin C	N	-0.6247	0.0028
CPA57_06190	flagellar hook-basal body complex protein FliE	N	-0.6253	0.0021
CPA57_03425	globin-coupled sensor protein	NT	-0.6335	0.0004
CPA57_06110	hypothetical protein		-0.6336	0.0027
CPA57_06400	flagellar hook-associated protein FlgK	N	-0.6661	0.0005
CPA57_06265	hypothetical protein	S	-0.6691	0.0011
CPA57_03200	hypothetical protein	V	-0.6740	0.0314
CPA57_06305	flagellar biosynthesis protein FlgA	N	-0.6805	0.0009
CPA57_06240	hypothetical protein		-0.6928	0.0009
CPA57_06275	flagellum-specific ATP synthase FliI	NU	-0.7184	0.0010
CPA57_06255	flagellar motor stator protein MotA	N	-0.7448	0.0019
CPA57_06350	hypothetical protein		-0.7578	0.0284
CPA57_06210	flagellar biosynthesis protein FlgE	N	-0.7683	0.0007
CPA57_06245	flagellar biosynthesis protein FlgD	N	-0.7767	0.0004
CPA57_06175	flagellar biosynthesis protein FlgB	N	-0.8101	0.0173
CPA57_06390	flagellar basal-body rod protein FlgG	N	-0.8238	0.0007
CPA57_07835	ABC transporter	V	-0.8283	0.0024
CPA57_06250	hypothetical protein		-0.8917	0.0020
CPA57_06300	chemotaxis protein	MNO	-0.9295	0.0084
CPA57_06820	histidine kinase	T	-0.9728	0.0356

CPA57_06170	flagellar basal body rod protein FlgC	N	-0.9790	0.0084
CPA57_03365	aconitate hydratase AcnA	C	-1.0065	0.0405
CPA57_06325	flagellar biosynthesis protein FlhA	N	-1.2276	0.0009
CPA57_06405	flagellar biosynthesis protein FlgL	N	-1.3197	0.0074
CPA57_07840	transporter	M	-1.3631	0.0032
CPA57_06395	flagellar basal-body rod protein FlgF	N	-1.3986	0.0039
CPA57_04515	hypothetical protein	U	-1.4708	0.0001
CPA57_07545	hypothetical protein		-1.6364	0.0033

Supplementary Data S8: GH 32 family enzyme ORFs of *B. apis* TMW 2.1884 with the locus tag DTI93_RS00530 in the genome NZ_QORR01000001.

>21884_B_apis_DTI93_RS00530

ATGACGTCGGATGATGATCGTGAGCCTTCCGGCAAGGATGTCTTCATCGACAAGAATAGCAGTCTTTCCGGCACGT
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TCGTCGATGATCATGGGAATACTCTGGGACATAAGTGGGCGATACTGGGAGCCGTGCAGGGTGATTATCCTGGTGG
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TCCGGCAGGTCTGAAGAGCAATGCGACTTCCAACATGATCATCACCATCCTGATCGATACCAGCAGCCTGGAAGTG
TTCATCAATGGAGGCCAGACGTCCCTGACAGGGCTGGTCTTTCCACCGAAGGGATGTACGGGGGTCAACATTACGT
CAACGCAGCCTGTTCATGTGTCCGTTTCGACAAGTGATTATTGA

<u>Extended version (additional bases are marked in bold):</u>

>21884_B_apis_ DTI93_RS00530_extended

ATGGTCACGATTCTTATTTTTGATAATATTTGCCGTAGATGGCACAATTTTTATGGCAACGCTCTCATGAGAC
GGGGCCATGTGTGCCCTATCGGTACTGTCATCGGATTTTTTCGCGAAAGGTTTTTGTCTATGACGGACCTGT
CCAATGTTTCGGTAGCGTATTATCCTAAGAGGCATCTGGCTCCTGTACCGGTCCCCGGCAGACACAAATGGC
AGAATGACGGTCAAAACTACGTTTATGACCGGAAGGCCAGACTGTGGAGGACGTGGGCTCTGGGAAACCCG
GAATGGACTCCTAACAGCGGTTTTCCAACGACGTCGTGGATTTCTTATAGCGGCCCTACCATCGATGCGATG
ACGTCGGATGATGATCGTGAGCCTTCCGGCAAGGATGTCTTCATCGACAAGAATAGCAGTCTTTCCGGCACGTACT
GGTCCGGAAGTGTGTGGGTGGATGAAGAAGACAGGCTGGGCCGTGGTCGGGGCAGTGTCTATTATTACATATCTGG
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GTCGATGATCATGGGAATACTCTGGGACATAAGTGGGCGATACTGGGAGCCGTGCAGGGTGATTATCCTGGTGGGA
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GGCCATCCCTCTGGATTACGGGCCGGATTCCTATGCGACCGTGGCAGGCCGCAATGGCAGGTCGACCTATGTGGGG
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GGCTTGCTGGATACAGACTGACTACAGTGGCCGGCAGAAAGTCTACACATGTCCTGTGGAAAAAGCGGATGGTATC
ACTGGGATAGGGGGCCCCAGGCAGACGATAGGTGGTGAGGGCAATCCTGATTTTGCGTCAGATGAGGTAGAGACG

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ATAAGGGCCGGGTGGAAGGCACGGAGTACAGTACCGACCTGATCATCTACCGGAGCGGTCAGATCACGTTTGACA
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GGCAGGTCTGAAGAGCAATGCGACTTCCAACATGATCATCACCATCCTGATCGATACCAGCAGCCTGGAAGTGTTC
ATCAATGGAGGCCAGACGTCCCTGACAGGGCTGGTCTTTCCACCGAAGGGATGTACGGGGGTCAACATTACGTCAA
CGCAGCCTGTTCATGTGTCCGTTTCGACAAGTGATTATTGA

9 List of publications and other contributions

Peer-reviewed publications (first authorships in bold)

1. **Härer, Luca**; Hilgarth, Maik; Ehrmann, Matthias A. (2022): Comparative Genomics of Acetic Acid Bacteria within the Genus *Bombella* in Light of Beehive Habitat Adaptation. In: *Microorganisms* 10 (5), S. 1058. DOI: 10.3390/microorganisms10051058.
2. **Härer, Luca**; Stýblová, Sabrina; Ehrmann, Matthias A. (2023): *Bombella pluederhausensis* sp. nov., *Bombella pollinis* sp. nov., *Bombella saccharophila* sp. nov. and *Bombella dulcis* sp. nov., four *Bombella* species isolated from the environment of the western honey bee *Apis mellifera*. In: *International journal of systematic and evolutionary microbiology* 73 (6), S. 5927. DOI: 10.1099/ijsem.0.005927.
3. **Härer, Luca**; Ernst, Luise; Bechtner, Julia; Wefers, Daniel; Ehrmann, Matthias A. (2023): Glycoside hydrolase family 32 enzymes from *Bombella* spp. catalyze the formation of high molecular weight fructans from sucrose. In: *Journal of Applied Microbiology* 134 (11). DOI: 10.1093/jambio/lxad268.

Scientific presentations (presenters in bold)

Härer, Luca; **Gaelings, Lana**; Kerpes, Roland; Becker, Thomas; Ehrmann, Matthias A. (2021): Nutzbarmachung osmotoleranter Starterkulturen aus Bienenhonig zur Entwicklung von Getränkezutaten auf Basis von Fruchtsaftkonzentraten. “Professorentreffen” of the Baumann-Gonser-Stiftung. Scientific presentation. Cologne, Germany.

Supervised student thesis

Stýblová, Sabrina (2022): Isolation and molecular characterization of new *Bombella* species, acetic acid bacteria with biotechnological potential. Master’s thesis.

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