Master thesis

to obtain the academic degree Dipl.-Ing.

Fructophilic Lactic Acid Bacteria: Characteristics and Safety Criteria

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Carried out at the

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Vienna, March 2018

ACKNOWLEDGEMENTS

To my supervisors,

Dr. Kneifel and Dr. Salminen, I express my sincerest gratitude for your patience and immense knowledge. Thank you Dr. Salminen for your accurate attention to detail. Dr. Kneifel, thank you for keeping me on track every time I had strayed.

To Dr. Akihito Endo,

Thank you for your achievements which have set the stage for most of the knowledge presented in this work, and thank you for taking the time to revise my thesis.

To my family,

Thank you for your unwavering love and support throughout my journey, for making all of this possible, and for your continuous motivation and enthusiasm.

Table of contents

Table of contents

Table of contents

1. Fructophilic Lactic Acid Bacteria

1.1 Origins and evolution

The Fructophilic Lactic Acid Bacteria (FLAB) are a sub-group of LAB (Endo and Okada, 2008). The main feature of the sources of FLAB is that they are fructose-rich. They have been found in edible sources such as fruits such as grapes, durian fruit, figs, banana and cocoa beans and legumes. They have also been found in fermented versions of fruits such as “tempoyak”, grape wines and musts, and palm tree sap wines. Moreover, they have been detected in different species of flowers and in the guts of different insects that consume fructose in high quantities. Such insects include bees, tropical fruit flies, *Camponotus* ants, and adult and larval honeybees. And more, they have been found in edible sources that are produced by honeybees such as honey and honey products (Endo *et al.,* 2012). There are, however, unanswered questions related to the exact origin of the FLAB, their evolution, and the dynamics of their populations in nature.

In general, at any given time, several factors determine the composition of a bacterial community. These factors are related to the host itself and to the environment that it lives in (Yun *et al*., 2014). The age and developmental stage of the host along with its phylogenetic identity determine the gut morphology and microbial identity, physicochemical conditions and oxygen demands, and the possible presence of a core microbiota. The environment and geographical location determine other characteristics such as the level of oxygen available, pH, the metabolites produced by accompanying bacteria or plants, and available food sources. The presence of oxygen is quite significant. Unlike oxygen, the effect of diet was not found to be as conclusive. In general, the diversity of bacteria in the guts of mammals and insects were found significantly higher in omnivores than in carnivores and herbivores. This can be explained by the fact that different food sources carry different sets of bacterial communities which are introduced into the gut upon consumption. However, this was not true for all groups of insects (Yun *et al*., 2014).

For example, FLAB have been identified in the nectar of flowers but it was not possible to differentiate whether they had been vectored from nectar sources or whether they have been transported onto them (Fridman *et al.,* 2012; Vojvodic *et al.,* 2013). Accepted theory suggests that the bacteria were already there and underwent an adaptive evolution. The presence of FLAB in such environments and their simultaneous inability to survive unaided under conditions that are normal for many other LAB are considered proof of evolutionary adaptation (Filannino *et al*., 2016). The symbiotic relationship observed between LAB, including FLAB, and bees which protects themselves and their host is referred to as colonization resistance (Vásquez *et al*., 2012). Environmental stresses threatening bees and their gut bacteria include excessive concentrations of fructose, high osmotic pressure, neighboring colonizing bacteria, bactericidal secondary plant metabolites, and active enzymes (Endo *et al*., 2015; Filannino *et al*., 2016). Such factors may be the drivers of the evolution of biochemical and physicological features of FLAB. Their biochemical properties are believed to have resulted from a reductive, fructophilic evolution (Endo *et al.,* 2012; Maeno *et al.,* 2017). The continued detection and dominance of FLAB in the same host sources suggests a coevolution and possible mutualistic relationship (Filannino *et al*., 2016).

Ultimately, gaining knowledge about the history, origins, genomics, and biochemical activities of FLAB is beneficial for the long term. Current knowledge is already explaining previously unknown anti-microbial properties of fresh, organic honey and other bee products which have been used for such purposes by the Mayans, in ancient Egypt, and in traditional medicine all over the world. It will facilitate the prediction of the possible responses to future challenges. These do not only include environmental concerns such as adaptability to climate change, but also the reactions with food matrices or performances in immunity-enhancing interventions (Silva *et al.,* 2017).

1.2 Overview of isolation and classification

The FLAB have been isolated from a myriad of sources since Endo and Okada suggested the formation of the particular group in 2008. Before then, the

corresponding bacteria were classified under the genera *Leuconostoc*, *Lactobacillus*, or were still unidentified as novel species. While the earliest characterized FLAB was isolated back in 1956, it was not until the turn of the century that the isolation and characterization of the remaining species progressed. A summary of the main identifiers recorded for all species of FLAB between the years 1956 and 2018 can be found in Tab. 1.

The analysis of small subunit rRNA gene sequences has promoted the reclassification of species in more suitable genera (Antunes *et al.,* 2002). The initially formed FLAB group was created as a sub-group of *Leuconostoc*. The four bacteria that made it up were reclassified as belonging to the novel genus *Fructobacillus* in the family of *Leuconostocaceae*. However, specific lactobacilli were later found to additionally belong to this group. Thus the FLAB are a group belonging to both families *Leuconostocaceae* and *Lactobacillaceae.* At the time of writing, eight species make up the FLAB. In chronological order of initial isolation, they are *Fructobacillus fructosus*, *Lactobacillus kunkeei*, *Fructobacillus ficulneus, Fructobacillus durionis, Fructobacillus pseudoficulneus, Lactobacillus florum, Fructobacillus tropaeoli,* and *Lactobacillus apinorum. L. florum* alone is classified as facultatively FLAB. The chronological sequence of events is displayed in Fig. 1 (see page 5).

The number of studies on FLAB has increased drastically after the year 2012. In 2008, Endo et al., proposed the use of fructose-containing media when isolating bacteria from fructose rich sources. In addition, more culture independent methods have been applied. These may have offset the previous underestimation of the numbers of FLAB in samples (Owens*,* 2014). What is sure is that interest in FLAB characterization and applications are continuously growing. There remains much to be investigated, but the knowledge available so far may help direct future research and decision-making. So far, applications in health promotion, product improvement, and chemical industry are some of the options being investigated (Endo, 2012; Asama *et al.,* 2015; Mayara *et al.,* 2017). It is realistic to expect that proper

utilization of FLAB may save lives and protect world economies through boosting immunity and process optimization.

Tab. 1: Overview of main identifiers of the FLAB species

Species	Year of isolation	Basonym	Natural sources	Isolation locations
Lactobacillus kunkeei	1998	*Lactobacillus kunkeei*	Wine, flowers, pollen honey, beebread bee GIT, bee hives	Chile, Bulgaria, Czech Republic, Italy, KSA, Panama, Sweden, Turkey, USA etc...
Lactobacillus florum	2010	*Lactobacillus florum*	Flowers Peony & Bietou, grapes & wine, select unripe fruits	South Africa, USA
Lactobacillus apinorum	2014	*Lactobacillus apinorum*	Honey stomach, fresh honey, bee bread	Sweden
Fructobacillus fructosus	1956	*Lactobacillus fructosus*	Flowers, musts & Taberna, honeybees	Spain & Mexico, South Africa, worldwide
Fructobacillus ficulneus	2002	*Leuconostoc ficulneum*	Figs, Tempranillo wine, cocoa beans	Spain, Ecuador
Fructobacillus durionis	2005	*Leuconostoc durionis*	Tempoyak, Taberna & Bandji palm wine	Mexico, Burkina Faso/West Africa, Indonesia & South East Asia
Fructobacillus pseudoficulneus	2006	*Leuconostoc pseudoficulneum*	Cocoa bean, ripe figs, banana, Taberna wine	Brazil/Ghana, South Africa, Mexico
Fructobacillus tropaeoli	2011	*Fructobacillus tropaeoli*	*Tropaeolum majus*, cocoa beans, Tempranillo wine, figs, bumble bees	Argentina Brazil/ Ecuador Malaysia, Spain

2. Genera and species and their natural sources

The members of the FLAB will be discussed in detail in this section. They will be organized by genus and then in order according to the year of isolation as shown in Fig. 2. As such, the *Lactobacillus* genus is discussed first because it was characterized earlier. The species are discussed starting with *L. kunkeei*, *L. florum*, and then *L. apinorum*. Then the *Fructobacillus* genus is discussed, starting with *F. fructosus*, *F. ficulneus*, *F. durionis*, *F. pseudoficulneus,* and finally *F. tropaeoli*. A strain of *L. fructivorans* has shown fructophilic properties and will be discussed in the Appendix I (see page 69).

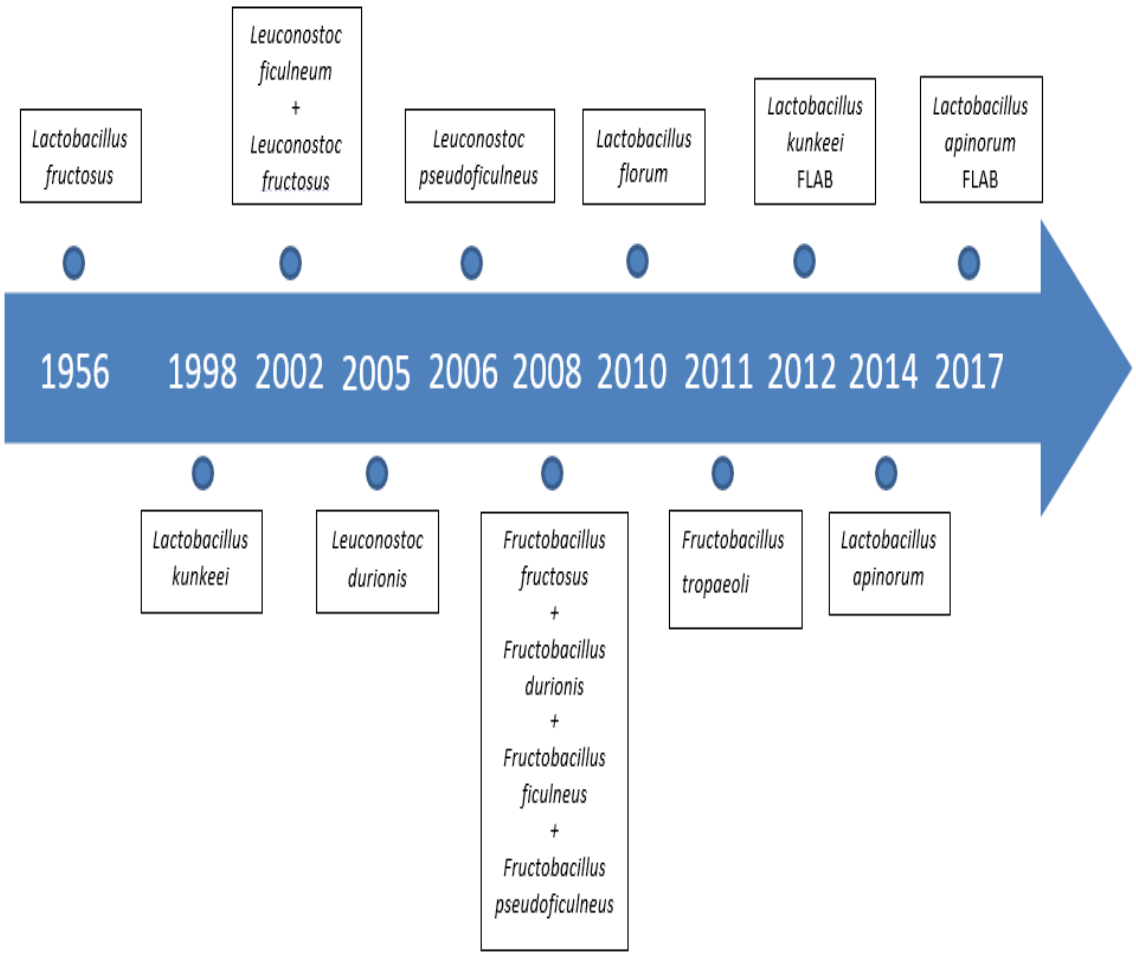

Fig. 1: Full timeline of FLAB isolation and classification

Genera, species, and their natural sources

2.1 *Lactobacillus*

2.1.1. *Lactobacillus kunkeei*

Lactobacillus kunkeei is the second oldest known FLAB. It was initially identified from a commercial Cabernet Sauvignon wine in 1998 by Edwards et al. It was named “kunkeei” after Dr. Ralph Kunkee as an appreciative gesture to his contributions to wine-related microbiology (Edwards *et al*., 1998). Since then, it has been isolated in flowers, honey, bee pollen, and beebread (Endo *et al.* 2009; Endo *et al.* 2012; Anderson *et al.* 2013; Anderson *et al.,* 2014; McFrederick *et al*., 2014; Asama *et al*., 2015; Tamarit *et al.,* 2015; Olofsson *et al.,* 2016a). Moreover, it was found in the gastrointestinal tracts of multiple insects including bumblebees, stingless bees, cross-continental species of honeybees, bumblebees, and halictid bees: *Megalopta centralis* and *Megalopta genalis* (Endo, 2012; McFrederick *et al.,* 2014; Hroncova *et al*., 2015; Lim *et al*., 2015; Uğraş, 2017). Perceptions surrounding it had been both positive and negative depending on the medium it is found in. This has led Bisson et al. (2017) to describe it as having two faces – one for wine spoilage and the other for probiotic properties. It is the most promising FLAB in terms of potential future benefit as its different strains are showing immunity boosting effects and anti-microbial properties against pathogens of insects, humans, and animals (Asama *et al.,* 2016; Berríos *et al.,* 2017).

Since the late 1990’s, *L. kunkeei* has been referred to as “ferocious lactobacilli” (Bisson *et al*., 2017). Fermentation is inhibited due to the high levels of acetic acid produced; the levels are similar to those produced by acetic acid bacteria (AAB). The type strain YH-15, in particular, has been considered especially notorious as it can inhibit *Saccharomyces bayanus* strain Prise de Mousse and *S. cerevisiae* strain Epernay which are two yeasts that are commonly used in the wine industry (Edwards *et al.,* 1998). Since *L. kunkeei* is quite abundant in the honeybee gastrointestinal tract, it may be unintentionally transported by bees into vineyards. The presence of *L. kunkeei* can lead to economic losses as it can cause fermentation arrest (Bisson *et al*., 2017). It is sensitive to naturally occurring sulfur dioxide (SO_2). However, SO_2 cannot be used to prevent fermentation arrest by *L.*

kunkeei because SO_2 itself is sensitive to other microbes present in the grape juice. Fermentation is stopped due to the high release of acetic and lactic acids and the release of inhibitory fatty acids and peptides. The low pH and inhibitory substances affect yeasts and also other sensitive competitive bacteria, two effects which are likely to prove beneficial in other applications (Bisson *et al*., 2017).

Besides in wine, different strains of *L. kunkeei* were isolated from different sources in other countries seemingly all over the world. The role it plays in bee immunity is being increasingly understood and is inspiring novel applications on humans and animals. Recent papers isolated *L. kunkeei* in honeybees in Bulgaria, the Czech Republic, Italy, the Kingdom of Saudi Arabia (KSA), Panama, Sweden, Turkey, and the United states of America (USA) (Olofsson and Vásquez, 2008; Hroncova *et al.,* 2015; Filannino *et al.,* 2016; Al Ghamdi *et al.,* 2017; Erban *et al*., 2017; Tonka Vasileva *et al.,* 2017; Uğraş, 2017). It was also found in bumblebees and halictid bees in the USA (McFrederick *et al.,* 2014; Lim *et al*., 2015). The same internationality applies to bee products because *L. kunkeei* was also isolated in flowers, honey, beebread, and pollen at least from Indonesia, Japan, Kenya, Malaysia, Mexico, Nepal, South Africa, Sweden, Thailand, and USA (Endo *et al.,* 2012; Anderson *et al.,* 2013; Anderson *et al*., 2014; McFrederick *et al*., 2014; Asama *et al*., 2015; Tamarit *et al.,* 2015; Olofsson *et al.,* 2016a). Additional information can be found in Appendix I (see pages 67-68).

For example, Anderson et al. (2014) performed a study on samples from honeybees' food which is stored in the hive and from the honeybees' digestive tract. The studies took place in Tucson, Arizona, USA. The focus was on the bacterial profile of beebread while seeking to find a link between behavioral or environmental factors and the nutrient composition of beebread. The study tests a commonly held, yet debated, scientific opinion about hive-stored pollen. It was hypothesized that it is continuous microbial succession that drives the creation of the stored pollen. This phenomenon leads to nutrient conversion which makes stored pollen an improved source of nutrition for bees (Anderson *et al.,* 2014). This hypothesis has been supported even though hive-stored pollen is highly acidic and contains high levels of

simple sugars making it unsuitable for the survival of many microbes (Anderson *et al.,* 2011). The results of the study by Anderson et al. (2014) disagreed with the aforementioned hypothesis; findings suggested that it is the addition of honey, nectar, and bee glandular secretions to pollen that make it a preservation substance. Regardless of the conclusion, *L. kunkeei* was indeed the only microbe to be isolated from the hive-stored pollen albeit in only four from sixty samples. The level decreases by only the fourth day. The reason behind the survival of *L. kunkeei* is thus contentious. It could be that it is contributing to the acidity of the pollen through fermentation metabolites. However, it may also be that this is the maximum duration of time that its adaptation abilities allowed it to survive (Anderson *et al.,* 2014). *L. kunkeei* is one of a small number of bacteria that were present during all seasons even if in limited amounts. As several other studies have reported, it is also abundant in the crop of adult honeybees and larvae as well as in honey and royal jelly. Therefore, *L. kunkeei* may contribute to the maintenance of the hive hygiene through the conservation of beebread and the safeguarding of larvae against pathogens (Anderson *et al.,* 2014).

Moreover, Olofsson et al. (2016a) in an attempt to identify the symbionts, bacteria living through mutually beneficial interactions, of honeybees that are responsible for the antimicrobial and therapeutic activity of honey also acknowledged the role of *L. kunkeei*. While not identified at the strain level, *L. kunkeei* Fhon2 was singled out in honeybee crops, honey, bee pollen and beebread. It was noted that *L. kunkeei* Fhon2 is always present but its abundance varied independently of honeybee species, geographic location of the bees or honey origin (Olofsson *et al.,* 2016a).

In addition, the seasonal difference in microbial abundance was touched upon by Anderson et al. (2014) and was different from the results of the study by Tamarit et al. (2015). *L. kunkeei* was also isolated in cultivation experiments in Helsingborg, Sweden. The report by Tamarit et al. (2015) is the first of its kind; a large-scale study analyzing and comparing genomes to *L. kunkeei* species. It was found dominant in samples of crop, honey, beebread, and pollen. Its abundance

was particularly noted during the spring and summer months whereas, it was nearly absent during the fall and winter (Tamarit *et al.,* 2015). In contrast, in the study performed in Arizona, USA, *L. kunkeei* was abundant in the fall and summer whereas, it was scarce during the winter and spring (Anderson *et al.,* 2014). These variations in results may well be due to the weather difference between the cold of Scandinavia and heat of Southern USA (Tamarit *et al.,* 2015).

Besides seasonal variation, the environmental influences were investigated by Vojvodic et al. in 2013. Samples of honeybee larvae were collected from two sites in Tucson, Arizona, USA. The larvae in either site differed in terms of whether they were managed (European) or non-managed (Africanized) bees. *F. fructosus* was among the less abundant isolates in managed European bees - which had access to flowers and crops. However, they were found in relatively significant amounts in non-managed Africanized bees - these were taken from a remote site in the Sonoran desert. The study also helped identify that royal jelly is a favorable growth medium for *F. fructosus.* An unexpected find is that royal jelly is normally considered to be inhibitory due to its chemical composition. Vojvodic et al. (2013) agree with previous studies that *L. kunkeei* and *F. fructosus* are not part of the core microbiota of adult bees, but their presence and abundance vary with yet unidentified environmental factors and favorable conditions.

In addition, Anderson et al. (2013) made a crucial observation in noting that *L. kunkeei* has been overestimated in samples from bee guts. When cloning or next generation sequencing methods were applied to the same samples as culturing techniques, results did not match. Instead, *L. kunkeei* was not often detected in samples of crop and hindgut. This comes in stark contrast to results obtained by culture-dependent methods. Therefore, they deduced that the proportions of *L. kunkeei* previously isolated were due to culturing bias. This observation was also supported by Filannino et al. (2016). However, the dominant presence of *L. kunkeei* in beebread was ascertained based on both culture-dependent and independent experiments (Anderson *et al.,* 2013).

Genera, species, and their natural sources

These studies led McFrederick et al. (2014) suggest that that the origin of *L. kunkeei* is not the bees themselves. Its abundance in the bee pollen and varying proportions in the guts presents additional proof that *L. kunkeei* is transferred horizontally, from the environment through the bees' social behavior and structure (McFrederick *et al.,* 2012; 2014).

Moreover, the status of flowers as sources of *L. kunkeei* was also contested (Tamarit *et al*., 2015). The nectar of certain flowers using 16S rRNA screening was not detected, but it was identified on the surface of honeybees visiting these same flowers. Thus, it has not been established whether flowers represent one of its growth niches, or whether *L. kunkeei* is only deposited in flowers through bee pollination (Tamarit *et al.,* 2015). It is also noteworthy that the ecological role of *L. kunkeei* within bees and beehives is not fully unknown yet. However, recent *in vitro* studies have shown that human and bee pathogenic bacteria and yeast can be inhibited by *L. kunkeei* (Tamarit *et al.,* 2015).

Focusing on bee genera and species, Tamarit et al. (2015) took samples from Indonesia, Kenya, Malaysia, Mexico, Nepal, Sweden, Thailand, and USA. The findings suggested that *L. kunkeei* is found internationally. Studies supporting of the internationality of *L. kunkeei* are plentiful. Different studies are attempting to understand the role that *L. kunkeei* plays, the mechanisms with which it can influence its environment, and the possibility of using it in novel applications.

To begin with, Filannino et al. (2016) analyzed samples from the guts of *Apis mellifera* L. collected from five different regions in Apulia, Italy. The purpose was to determine whether phenolic acids are efficient external electron acceptors during the metabolism of glucose by FLAB. *F. fructosus* merely made up 31% of the total isolates whereas, *L. kunkeei* accounted for 61%. It was found that, of the phenolic acids, p-coumaric acid may be used as an electron acceptor, but not as efficiently as fructose and pyruvic acid (Filannino *et al.,* 2016). Such knowledge is necessary to facilitate the choice of suitable media and conditions in which to introduce *L. kunkeei*. The protection from diseases of bee hives, bee feed, flowers and plants,

larval colonies, human GI tracts and immune systems are some of the possible objectives of future applications.

The aforementioned aims are being investigated in studies from around the world. For example, in the KSA, *F. fructosus* and *L. kunkeei* were isolated from the indigenous honeybees of the KSA which were kept at the King Saud University, Riyadh, Saudi Arabia (Al Ghamdi *et al.,* 2017). The aim of the study was to investigate the probiotic effects of *F. fructosus* and *L. kunkeei* on honeybee larvae infected with *Paenibacillus larvae*. Each of the isolated bacteria was added individually to the diet of the larvae and the mortality was noted over six days. Results showed that *L. kunkeei* was the single most effective treatment. Mortality after six days was 56.67% and 78.33% for *L. kunkeei* and *F. fructosus* respectively compared to 86.67% of the positive control (Al Ghamdi *et al.,* 2017). This study further supports the growing consensus that *L. kunkeei* has probiotic potential. Whether it is best administered on its own or as part of a combination is uncertain as of yet, but other studies have shown that *L. kunkeei* can be used as part of a group of symbionts exhibiting synergistic relationships (Olofsson *et al.,* 2016a).

Moreover, a recent study in Düzce, Turkey investigated whether it is possible to use probiotic bacteria such as *L. kunkeei* against antibiotic-resistant bee diseases (Uğraş, 2017). This study is the first time that *Lactobacillus kunkeei* was isolated from the honey stomach of the Turkish Yigilca honeybee (Uğraş, 2017). The inhibitory activity of a number of isolated strains was evaluated against a number of indicator bacteria. The strain *L. kunkeei* HD1 was the most promising even though it did exhibit hemolytic activity, rasing safety concerns related to possible ingestion by animals and humans, and was found resistant to two of the eight tested antibiotics. Regardless of this, it was able to inhibit most of the indicator bacteria especially *Melissococcus plutonius* which is the cause of the European foulbrood (EFB) disease. Further research is needed, but the foundations are encouraging. The study suggests taking preventative actions that eliminate the need for treating beehives with antibiotics. The development of preparations of such probiotic

bacterial isolates may be successful in improving the immunity of the hive if directly administered by the beekeepers (Uğraş, 2017).

In addition, an outbreak of EFB hit the Krkonose Mountains National Park in the Czech Republic in 2015 after almost forty years of absence (Erban *et al*., 2017). EFB is caused by the bacteria *Melissococcus plutonius.* Therefore, it became necessary to study its impact on the microbiome of the worker bee. A study by Erban et al. (2017) classified samples as EFB0 meaning fully asymptomatic, EFB1 meaning clinically asymptomatic bees but the apiary shows signs of EFB, and EFB2 representing observed clinical symptoms. A total of 49 samples consisting of ten surface-sterilized worker bees from twenty seven honeybee colonies were taken. Results found that *F. fructosus* and *L. kunkeei* are significantly higher in EFB2 than in EFB1. The presence of EFB appears directly related to the changes in the microbiome. This led to the theory that they might possess immunity-related functions. While *L. kunkeei* is believed to protect the honeybee against *M. plutonius*, the function is not usually attributed to *F. fructosus* (Endo and Salminen, 2013; Vásquez *et al.,* 2012). Occasionally, the proportions of *F. fructosus* have been found to emulate those of *L. kunkeei* based on a METASTATS analysis. Erban et al. (2017) postulated that dietary composition and/or processing changes in honeybees could have led to the observed changes in proportions of the different bacterial taxa. Besides honeybees, *L. kunkeei* was also isolated in various species of bumblebees, stingless bees, and halictid bees while maintaining support for the global character.

To start with, a study by Lim et al. (2015) aimed to characterize the gut microbial communities of nine bumblebee species in the USA. Findings indicated that bee species as well as geographic location were greatly influential, thus supporting the previously noted theory of horizontal transmission of bacterial species. *Fructobacillus tropaeoli* and *Lactobacillus kunkeei* were among the six most dominant isolates, but they were not identified at strain level (Lim *et al*., 2015).

Moreover, *L. kunkeei* was isolated in stingless bees in Mexico and Kenya (Vásquez *et al.,* 2012). Its dominance in Central American *Melipona beecheii* species and the African *Meliponula bocandei* was evaluated. However, it was not

detected in *Trigona sp.,* the stingless bees of Borneo, Malaysia or Thailand. It was found that *L. kunkeei* and similar bacteria which show antimicrobial properties are transmitted among nest-mates and are maintained within the crop in biofilms. It was also found that beekeeping practices may harm or reinforce the microbial communities through the supplementation of the feed and other prophylactic practices. It is expected that *L. kunkeei* is going to be central to the attempts to combat Colony Collapse Disorder (CCD) (Vásquez *et al.,* 2012).

Finally, McFrederick et al. (2014) studied the halictid bee species *Megalopta centralis* and *M. genalis*. They are facultatively social sweat bees meaning that, unlike honey- and bumblebees, they are primarily solitary and primitively eusocial. Because *L. kunkeei* is an environmentally acquired microbe, it was common to all *Megalopta* bacterial communities. Findings indicate that the environmental transmission compensates for the suboptimal social transmission. This is because the diet of *Megalopta centralis* and *M. genalis* revolves around a limited number of plant species (McFrederick *et al*., 2014). The question remains for whether it is predominantly bee- or flower- or pollen-associated or if it is capable of thriving in all three equally (McFrederick *et al*., 2014).

Recent studies have focused on closely investigating the efficiency and likelihood of using *L. kunkeei* in strengthening human immunity. Asama et al. (2015) published the first trials on humans; the first one done in Japan found that the heat-treated *L. kunkeei* YB38 strain successfully increases secretory IgA (SIgA) concentrations. The second study became the first trial to investigate the effect of heat-treated *L. kunkeei* YB38 on the human intestinal tract and on bowel movement (2016). The results were favorable on both accounts (Asama *et al.,* 2016). On a similar note, the ability of *L. kunkeei* to produce biofilms that attenuate infections of *Pseudomonas aeruginosa* was tested. The study was performed *in vitro* and on *Galleria mellonella* honeycomb moth larvae.The results showed some positive effect but positive results were limited and strain-specific (Berríos *et al.,* 2017). The mechanism is postulated to be either through improving the immunity of the *G. mellonella* or by affecting the *P. aeruginosa* through anti-microbial genes (*Berríos et*

al., 2017). More trials and research are required on all fronts, but the preliminary signs are encouraging.

Finally, the widespread presence of *L. kunkeei* and its main role in bee immunity make it a desirable candidate for paratransgenesis studies (Maddaloni *et al.,* 2014). If successful, its applications can be diverse and thus solve more than one problem in one go. Unfortunately, though, *L. kunkeei* did not satisfy the requirements during experimentation. The main shortcoming of *L. kunkeei* is that the cells tended to flocculate in rather large clusters. Attempted pipetting or sonication to disrupt the clusters mostly failed and has even caused cellular contents to be released. Such difficulties have left *F. fructosus* as the more likely option (Maddaloni *et al.,* 2014).

2.1.2. *Lactobacillus florum*

Lactobacillus florum was initially isolated from Peony (*Paeonia suffruticosa*) and Bietou (*Chrysanthemoides monilifera*) flowers in South Africa (Endo *et al*., 2010). It has also been encountered in South African grapes and wine as well as in unripe king palm fruit, cactus, rose apple, tangelo, and Valencia orange leaf in California (Endo *et al.,* 2010; Mtshali *et al*., 2012; Kim *et al*., 2013; Tyler *et al.,* 2016). The isolated strains showed high genetic similarity allowing the conclusion that they all belong to the same species. However, the DNA fingerprints indicated differences at the strain level (Endo *et al*., 2010). Two of the *L. florum* strains, 8D and 2F, do stand out, but the role of the species is not yet fully clear in all the sources it has been identified in. So far, alongside lactate, acetate, and ethanol, there is evidence of the production of polyols – namely, mannitol and erythritol. Studies performed by Tyler et al. (2016) showed that the substrates used and combinations thereof affected the growth rates of the bacteria as well as the products and yield of the fermentation. More specifically, the strain 2F fermented fructose into mannitol, but it produced erythritol from glucose. Moreover, the strain 8D produces erythritol in higher amounts than 2F (Thomas, 2015). The predicted metabolic pathway resulting in mannitol production from *Fructobacillus* spp. can be found in the Appendix I (see page 80).

Genera, species, and their natural sources

It became the first species to be classified as facultative FLAB because the characteristics it exhibits are mainly similar to those of FLAB except for some biochemical differences (Endo *et al.,* 2010). For example, besides D-fructose, it only fermented D-glucose, albeit much slower, from 49 carbohydrates that were tested. In contrast to other FLAB, *L. florum* could ferment D-glucose in the absence of an electron acceptor even though its presence did enhance growth. Also, the ratios of the dissimilation of the sugars did not fit other FLAB. Similarly to FLAB, growth rates and yields were higher when both fructose and glucose were fermented (Tyler *et al*., 2016). As such, it became the first facultatively FLAB species (Endo *et al*., 2012). Moreover, the 16S rRNA gene sequence revealed that the *L. florum* strains formed a subcluster with *Lactobacillus buchneri* and were specifically closely related to *Lactobacillus lindneri* (Endo *et al*., 2010). Yet, the strains could still be clearly differentiated from *L. buchneri* and the other related species.

Mtshali et al. (2012) developed a novel PCR assay in order to identify *L. florum*. The end goal is to find genes that code for oenologically relevant enzymes. This would allow the inference of whether the presence of the species has a positive or negative impact depending on its surrounding matrix. The PCR results included the detection of genes encoding for peptidases, for an incomplete arginine deiminase pathway, for a phenolic acid decarboxylase enzyme, and of citrate lyase genes (Mtshali *et al*., 2012).

These results indicate that the strains may play a role in peptidolysis, but the role of wine LAB in peptide formation is not fully understood. Also, the arginine deiminase pathway in the form that it was found may lead to the formation of urethane. Urethane does not only possess negative health effects, but it is also potentially carcinogenic (Ough *et al*., 1988). Moreover, it is the phenolic acid decarboxylase enzyme which allows the formation of volatile phenols in wine (Liu, 2002; Mtshali *et al.,* 2012). Also, the existence of citrate lyase genes which is relevant to the production of the buttery flavor compound diacetyl. In appropriate amounts, the buttery flavor may be pleasant, but it is considered undesirable beyond a certain limit (Liu, 2002; Fornachon and Lloyd, 1965; Rankine *et al*., 1969; Mtshali

et al., 2012). However, some genes involved in the citrate pathway are absent which means that it may not be active. Finally, the results also suggest that the different strains do not produce hazardous biogenic amines (Smit *et al*., 2008; Mtshali *et al*., 2012).

It is important to note, though, that false positives are possible - especially in the case of the genes relevant to the citrate pathway (Mtshali *et al*., 2012). Such results would be obtained if the PCR primers failed to bind to and amplify the fragment of the relevant gene. Therefore, follow-up studies are required (Mtshali *et al*., 2012). Also, the study by Tyler et al. (2016) on the gene and protein homology of *L. florum* 2F succeeded in identifying the genes coding for mannitol dehydrogenase enzyme, but they failed to find those responsible for erythritol biosynthesis.

2.1.3. *Lactobacillus apinorum*

The most recently isolated species of FLAB is *Lactobacillus apinorum*. It was isolated as one of seven novel species from the honey stomach of the *Apis mellifera* honeybee by Olofsson et al. in 2014. From older studies, the microbiota was correctly expected to consist of phylotypes of genera *Lactobacillus* and *Bifidobacterium.* Upon its isolation, *L. apinorum* was given its name after the Apini tribe of honeybees which only consists of bees of the "*Apis*" genus. The closest type strain to *L. apinorum* is *Lactobacillus kunkeei* as the 16S rRNA gene sequence similarity is 98.9% (Olofsson *et al*., 2014).

Maeno et al. (2017) performed a study on the fructophilic characteristics of *L. apinorum* given the likelihood that it is a FLAB. Relevant factors are that it is commonly found in fructose-rich sources, and it only produces acids from D-fructose alone or from the fermentation of both D-glucose and D-fructose together. Also, it was found to lack the enzyme alcohol/acetaldehyde dehydrogenase gene (adhE), and it exhibits high 16S RNA gene sequence similarity to *L. kunkeei* (Endo *et al.,* 2014; Olofsson *et al*., 2014; Maeno *et al.,* 2016). Therefore, more biochemical and genomic characteristics were tested. These tests examined additionally the functional gene content profile, the activity of NADH oxidase and the absence of a

phosphotransferase system (PTS) and terpenoid-quinone biosynthesis and compared them to other LAB and FLAB. The results led to the classification of *L. apinorum* as an obligate FLAB species belonging to the *Lactobacillus* genus (Maeno *et al.,* 2016).

A comparison of the encountered strains with GenBank worldwide database entries revealed that both similar and different strains of LAB have also been isolated from other sources. The strains which were found to have 100 % similarity mostly had the same or very close sequence length. The sources of the strains in question include fresh honey from *A. mellifera Buckfast*, bee bread from *A. mellifera scutellata*, and honey stomach from *A. mellifera mellifera*, *A. mellifera*, *A. koshevnikovi*, *A. cerana*, *A. nuluensis*, and *A. laboriosa* (Olofsson *et al*., 2014).

Therefore, the microbiota inhabiting the aforementioned sources is fundamental in the production and preservation of food by the bee (Olofsson *et al*., 2014). And more, it has demonstrated protective action for bees against pathogens which may attack the bee directly or be naturally occurring in the nectar (Vásquez *et al*., 2012). As such, *L. apinorum* in combination with *L. kunkeei* and others may have the potential to for beneficial future applications. Some of the recently submitted patenting cases include their use as nutraceutical supplements in bee probiotics, bee hive immunity enhancing sprays, and therapeutic skin creams for humans. These will be discussed in the applications section and in the Appendix I (see pages 70-74).

2.2 *Fructobacillus*

2.2.1. *Fructobacillus fructosus*

Fructobacillus fructosus was initially isolated from flowers by Kodama in Japan in 1956 as *Lactobacillus fructosus* pertaining to fructose (Endo and Okada 2008). It has been reclassified twice since - first as *Leuconostoc fructosum* in 2002 and then as *Fructobacillus fructosus* in 2008 (Antunes *et al*., 2002; Endo and Okada, 2008). In 2002, due to similar cell morphology and biochemical characteristics to the novel strain at the time, *Leuconostoc ficulneum*, it was

classified as *Leuconostoc* (Antunes *et al.,* 2002). Then, in 2008, Endo and Okada proposed its second reclassification after a number of *Leuconostoc* species were found to form a notably different sub-clubster from the rest of the genus constituents – *F. frustosus* was made the type species. It had not been reported over the next fifty years until 2010 when it was identified in Azalea flowers in South Africa and then in taberna palm sap wine in Mexico (Endo *et al*., 2009; Alcantara-Hernandez *et al.,* 2010; Endo, 2012). It was isolated again in 2011 in musts from Spain (Mesas *et al.,* 2011). Recently, it has been isolated from a number of insects. Research on a number of species of bees, ants, and tropical fruit flies has identified *F. fructosus* amongst the corresponding bacterial profiles. It was found in *Camponotus* ants in China and tropical fruit flies in Australia (Thaochan *et al.,* 2010; He *et al.,* 2011; 2014). Moreover, it being regularly isolated in guts of worker honeybees from different countries including the Czech Republic, Italy, the KSA, and the USA (Endo and Salminen, 2013; Vojvodic *et al.,* 2013; Filannino *et al*., 2016; Al Ghamdi *et al.,* 2017; Erban *et al*., 2017). The role and functions played by *F. fructosus* give an indication of possible future applications. So far, it may be used as a starter culture for fermentation, in the food and chemical industries through mannitol production, in combination with other bacteria as a bee probiotic, and/or can be used to protect bees from threats of diseases through genetic modification. More research is required regarding its role in ants and fruit flies.

As previously mentioned, *F. fructosus* was detected alongside *F. durionis* in Mexican taberna coyol sap wine. Altogether, 15 samples were collected from traditional producers in Chiapas, southern Mexico. Preliminary analysis was completed on pH, total acidity, and sugar content. The samples were thereafter fermented at room temperature with DNA analysis performed at 0, 60, and 108hrs. *F. fructosus* and *F. durionis* were identified only in the initial stages of the fermentation. Both species were no longer isolated later during the fermentation processes (Alcantara-Hernandez *et al*., 2010).

In another case, a study by Mesas et al. (2011) took place over three consecutive years across different parts of the Ribeira Sacra region in north-west

Spain. Samples of musts, the earliest form of wine, and of wines from four cellars were taken and species were identified according to recommended methods. *F. fructosus* and *L. kunkeei* were both isolated and were significantly abundant in musts; 13 strains of *F. fructosus* and 24 strains of *L. kunkeei* were identified. However, they were not present in the corresponding final wine products (Mesas *et al*., 2011).

The noted absences in the final wine and sap wine products indicate the loss of suitable survival conditions. *F. fructosus* can only ferment fructose, mannitol, and glucose in the presence of an electron acceptor (Antunes *et al*., 2002). Another reason could be the development of conditions in which they cannot survive. This can be related to the unavailability of substrates, inadequate pH levels, unsuitable growth temperatures, or from the effect of other bacterial species that hindered the survival of the FLAB species (Antunes *et al.,* 2002; Alcantara-Hernandez *et al*., 2010).

On a different note, research on insects such as *Camponotus* ants and tropical fruit flies using molecular identification methods managed to isolate *F. fructosus* among a number of other uncommonly isolated bacteria (Thaochan *et al*., 2010; He *et al.,* 2011; 2014). Fewer studies have been documented on these insects than on bee species. Therefore, while the current information sheds some light, more studies are needed in order to reach conclusions.

F. fructosus has been isolated in fruit flies. A study was performed in 2007 in Brisbane, Australia by Thaochan et al. (2010) on *Bactrocera cacuminata* (Hering) and *Bactrocera tryoni* (Froggatt) flies. The flies collected were hand-picked, adults. During sample collection, the *B. cacuminata* flies were taken out of wild tobacco, *Solanum mauritianum Scopoli*. *B. tryoni* were collected from tropical fruits such as custard apple (*Annona reticulata L*.), guava (*Psidium guajava L*.) and loquat (*Eriobotrya japonica* (Thunberg) (Lindl.). Special measures were taken to protect the flies and prevent any unintentional exchange of bacteria between the specimens. Both API 20-E and molecular cloning were performed on the 16S rRNA gene from the crop and midgut of the flies' GI tract. The API 20-E only identified one family –

Enterobacteriaceae – because it was limited by the use of the tryptic soy agar (TSA) and peptone yeast extract agar (PYEA) media. The molecular cloning method, however, managed to identify many other bacteria. The *Firmicutes* phylum was the predominant one detected. It was noted that the clones obtained from the crop area were mainly Gram positive bacteria regardless of the species of the fruit fly. It was also found that Gram-positive bacteria were found in the midgut at a considerably lower proportion than in the crop. While LAB were found in both fly species, they were more common in *B. cacuminata* (90.48%) than *B. tryoni* (52.63%). This study represented the first account of LAB in *Bactrocera* fruit flies. The genera *Lactobacillus, Leuconostoc, Pediococcus* and *Vagococcus* were present, and *F. fructosus* was one of the most commonly represented of the Firmicutes clones (Thaochan *et al.*, 2010).

Over the course of the next few years, studies focused on the microbial profile of different species of *Camponotus* ants (He *et al*., 2014). Considering that *Camponotus* is the second largest ant genus, studies related to bacterial presence can help differentiate between commensals and members of a core gut microbiota. *F. fructosus* was only identified in *Camponotus japonicus Mayr* and not in in *Camponotus fragilis* (He *et al*., 2011; 2014).

The earlier published study centered on the microbiota of the gut of the *Camponotus japonicus Mayr.* The sample collection took place in August, 2009 at the campus of the Northwest A&F University in the Shaanxi province in China (He *et al*., 2011). Foraging worker ants were collected and underwent gut 16S rRNAs polymerase chain reaction (PCR)-restriction fragment-length polymorphism analysis. The resulting clones belonged to the four bacteria *Candidatus Blochmannia*, *Candidatus Serratia symbiotica*, *F. fructosus,* and an uncultured *Burkholderiales* bacterium - noted here respectively in order of predominance. *F. fructosus,* and the *Burkholderiales* bacterium only made up 2% of the total clones. A low diversity of *C. japonicas* gut microbiota was also noted. Gastrointestinal tracts of other species and other age-stages were found to have different bacterial profiles. For example, a

previous study in 2007 by Feldhaar et al. using TGGE community fingerprinting only identified *Candidatus Blochmannia* in *C. floridanus* guts (He *et al*., 2011).

The relationship between the variability of the gut microbiota and the environment and corresponding colonies has not been established. However, it is suggested by He *et al.,* that the differing diets lead to accordingly different gut microbial communities which in turn provide specific benefits to their host species. The food sources of *C. japonicus* were either honeydew from nearby Cypress trees or foraged food from the ground. Consumed nectar and honeydew are the likely sources of *F. fructosus* to the *C. japonicus* ant diet. At the time, *F. fructosus* was not yet identified as having some probiotic properties. However, He et al. (2011) did propose the possibility that it contributes to sugar digestion based on its fructophilic properties.

On the other hand, results of the later published study did not identify *F. fructosus* in the *Camponotus fragilis* species (He *et al*., 2014). The study included both lab-originating and field-originating ants. The specimens of the first group consisted of lab-raised *C. fragilis* and were collected from Tucson, Arizona in June 2009. The collection of the second group took place in September 2010. It consisted of field-raised foraging workers and was collected from two sites. The first site was Staghorn Chollas at the Saguaro National Park East in Tucson, Arizona. The second site was Pajarita Mountains in Santa Cruz County, Arizona. The analysis included both microbial culturing and a molecular technique which is 16S rRNA-RFLP. Lab raised ants showed a greater gut microbial diversity with *Bacillus*, *Staphylococcus*, and *Blochmannia* being the only bacteria in common. He et al. (2014) postulated that ants do not share a core gut microbiota the way honeybees have been found to. This is significant because a core microbiota implies a fixed beneficial function by its constituting members, whereas commensals usually have little effect on the host or any particularly harmful pathogens (He *et al*., 2014).

On a wider scale, studies on different species of honeybees and for different purposes took place in different countries all over the world. A common feature among these studies was the isolation of *F. fructosus* and *L. kunkeei*. They were

prominent species in the intestinal contents of worker honeybees in multiple studies –although *F. fructosus* was detected in fewer geographical locations and on a smaller scale (Endo and Salminen, 2013; Vojvodic *et al*., 2013; Filannino *et al*., 2016; Al Ghamdi *et al*., 2017).

Specifically, Filannino et al. (2016) analyzed samples from the guts of *Apis mellifera* L. collected from five different regions in Apulia, Italy. The purpose was to determine whether phenolic acids are efficient external electron acceptors during the metabolism of glucose by FLAB. *F. fructosus* merely made up 31% of the total isolates whereas *L. kunkeei* accounted for 61%. It was found that, of the phenolic acids, p-coumaric acid may be used as an electron acceptor, but not as efficiently as fructose and pyruvic acid (Filannino *et al.,* 2016).

Similarly, in the KSA, *F. fructosus* and *L. kunkeei* were isolated from the indigenous honeybees of the KSA which were kept at the King Saud University, Riyadh, Saudi Arabia (Al Ghamdi *et al.,* 2017). The aim of the study was to investigate the probiotic effects of *F. fructosus* and *L. kunkeei* on honeybee larvae infected with *Paenibacillus larvae*. Each of the isolated bacteria was added individually to the diet of the larvae and the mortality was noted over six days. Mortality after six days was 56.67% and 78.33% for *L. kunkeei* and *F. fructosus* respectively compared to 86.67% of the positive control (Al Ghamdi *et al.,* 2017). Therefore, *F. fructosus* on its own did have some beneficial effect. While the benefit is not fully clear and undeniable, potential probiotic applications as part of a combination can be explored next.

And more, *F. fructosus* and *L. kunkeei* were found together in honeybees in the USA (Vojvodic *et al.,* 2013). Samples of honeybee larvae were collected from two sites in Tucson, Arizona, USA. The larvae in either site differed in terms of whether they were managed (European) or non-managed (Africanized) bees. *F. fructosus* were among the less abundant isolates in managed European bees - which had access to agriculture. However, they were found in relatively significant amounts in non-managed Africanized bees - these were taken from a remote site in the Sonoran desert. The study also helped to identify royal jelly as a favorable

medium of growth for *F. fructosus.* An unexpected finding is that royal jelly is normally considered to be inhibitory due to its chemical composition. Vojvodic et al. (2013) agree with previous studies that *L. kunkeei* and *F. fructosus* are not part of the core microbiota of adult bees, but their presence and abundance rather vary with yet unidentified environmental factors and favorable conditions.

In addition, an outbreak of European foulbrood (EFB) hit the Krkonose Mountains National Park in the Czech Republic in 2015 after almost forty years of absence (Erban *et al*., 2017). EFB is caused by the bacteria *Melissococcus plutonius.* Therefore, it became necessary to study its impact on the microbiome of the worker bee. A study by Erban et al. (2017) classified samples as EFB0 meaning fully asymptomatic, EFB1 meaning clinically asymptomatic bees but the apiary shows signs of EFB, and EFB2 representing observed clinical symptoms. A total of forty-nine samples consisting of ten surface-sterilized worker bees from twenty seven honeybee colonies were taken. DNA extraction was performed using conventional PCR and by analysis of the 16S rRNA genes using Illumina MiSeq amplicon sequencing. Results indicated that *F. fructosus* and *L. kunkeei* are significantly higher in EFB2 than in EFB1. The presence of EFB appears directly related to the changes in the microbiome. This led to the theory that they might possess immunity-related functions. While *L. kunkeei* is believed to help protect the honeybee against *M. plutonius*, the function is not usually attributed to *F. fructosus* (Endo & Salminen, 2013; Vásquez *et al.,* 2012). However, the proportions of *F. fructosus* have been found to emulate those of *L. kunkeei* based on a METASTATS analysis. Erban *et al.* postulated that dietary composition and/or processing changes in honeybees could have led to the observed changes in proportions of the different bacterial taxa (Erban *et al.,* 2017).

Furthermore, a feature of *F. fructosus* is its notable ability to produce mannitol. *F. fructosus* showed the highest volumetric productivity and the highest yield among eight mannitol-producing bacteria. It consumed both fructose and glucose simultaneously, but the rate of fructose assimilation was always higher. In addition to mannitol, it also produces lactic acid, acetic acid, and small amounts of

erythritol (Antunes *et al.,* 2002; Carvalheiro *et al*., 2011)**.** Therefore, *F. fructosus* can be considered a candidate for more efficient mannitol production for use in the food or chemical industries.

Finally, its use as a genetically modified microorganism (GMM) was proposed by Maddaloni et al. in 2014. GMM's are a focus of controversy and are additionally complicated by the limitations imposed by the European Commission. The study by Maddaloni et al. (2014) was the first study of its kind on FLAB. It serves to lay the groundwork and tools for paratransgenesis of honeybees. The definition of paratransgenesis from Hurwitz et al. (2011) is that it is "the process of altering the host's microbiome by introducing a genetically engineered microorganism" (Maddaloni *et al*., 2014, p. 2). The goal is to eliminate or limit disease spread which in the case of honeybees could save them from dramatic losses and possible extinction. This could simultaneously save the environment from the dramatic economic, agricultural, and ecological costs. In these studies, *F. fructosus* was considered a better candidate for paratransgenesis than *L. kunkeei* because of its efficient transformation properties. Still, it was generally accepted that both species are amenable to extensive genetic modification. However, such a project necessitates and requires extensive further research and trial applications (Maddaloni *et al*., 2014). This point will be elaborated further in the applications section.

2.2.2. *Fructobacillus ficulneus*

In 2002, Antunes et al. isolated the novel species *Leuconostoc ficulneum* from ripe figs. Its closest relative was determined to be *Lactobacillus fructosus* from phylogenetic analysis of 16S rRNA genes and DNA-DNA re-association values. Growth and phenotypic characteristics as well as fatty acid composition differentiated *L. ficulneum* from *Lactobacillus fructosus.* However, both organisms were found to only ferment glucose when it is in the presence of fructose, but they had the ability to ferment fructose alone (Antunes *et al*., 2002). As previously mentioned, the taxonomic study performed by Endo and Okada (2008) on the *Leuconostoc* genus led to the formation of the novel genus *Fructobacillus* and to the

reclassification of four *Leuconostoc* species; thus, *Leuconostoc ficulneum* was renamed *Fructobacillus ficulneus*. The main role observed by *F. ficulneus* is that it takes a minor part in fermentations and is an avid polyol producer especially of mannitol and acetate while lactate and erythritol are produced in smaller amounts.

Fructobacillus ficulneus was isolated from ripe figs and, surprisingly, in Tempranillo red wine from Rioja, Spain (Endo *et al.,* 2012; González-Arenzana *et al.,* 2015). It was thought that *F. ficulneus* has only ever been identified in figs (Endo *et al*., 2012). However, it has been unexpectedly encountered in two other cases more recently. In 2011, Papalexandratou et al. performed four spontaneous fermentations in the traditional way on cocoa beans from hybrid Nacional × Trinitario trees from Mocache, Ecuador. *F. ficulneus* was identified in the first round of fermentation only as a minor species, but it did not reappear. The experiment focused on the effects of the different traditional techniques of fermentation of cocoa beans at the microbiological and chemical level as affected additionally by environmental influences. It revealed how such uncontrolled conditions influence the microbial profile whose metabolites can negatively impact the further production steps. Conditions for the uncharacteristic growth of *F. ficulneus* became favorable temporarily, and it disappeared once the conditions were no longer met (Papalexandratou *et al*., 2011).

On the other hand, a study by González-Arenzana et al. on Tempranillo red wine from the Rioja, Spain region started in 2006. In 2012, González-Arenzana et al. investigated the ecology of indigenous LAB along wine making processes as well as their interactions in two publications. In 2015, a study on the efficiency of analysis of alcoholic and malolactic fermentation by LAB using culture dependent compared to independent methods was published (González-Arenzana *et al.,* 2015). In order to do this, samples of Tempranillo red wine were investigated from the 2006, 2007, and 2008 vintages from a winery in Rioja. Most recently, González-Arenzana et al. (2017) looked into the effectiveness of culture independent methods such as electrophoresis and PCR compared to plating techniques. Results by PCR-16S rDNA-DGGE identified *F. ficulneus* from wine for the first time in two out of three

years as opposed to plating techniques. In general, culture-independent methods detected more or the same number of LAB species as plating. Only rarely did plating identify more species (González-Arenzana *et al*., 2015).

Like other FLAB, *F. ficulneus* grows in media where glucose is not the only carbon source - as long as the media do not contain more than 40% glucose. API 50CHL microtubes show that it ferments the D-glucose and D-fructose well, but it is slow in fermenting gluconate, mannitol and trehalose and weak in fermenting sucrose and D-turanose (Antunes *et al.,* 2002). It is important to note that the assimilation rate of fructose is always higher than that of glucose (Carvalheiro *et al*., 2011). From [3-^{13}C] fructose, *F. ficulneus* mainly produces mannitol, acetate and lactate to a smaller extent, and erythritol in smaller amounts (Antunes *et al.,* 2002). A study aimed at evaluating the mannitol producing capabilities of different bacteria classified *F. ficulneus* in the high yield category ranging between 1.41 - 1.89 g/l h (Carvalheiro *et al*., 2011).

2.2.3. *Fructobacillus durionis*

Fructobacillus durionis is one of the species of FLAB about which the least is known. At the time of isolation in 2005, the species was classified as *Leuconostoc* following a 98% phylogenetic sequence similarity to the *Leuconostoc fructosum* (Endo and Okada, 2008; Leisner *et al*., 2005). In 2008, Endo and Okada performed a taxonomic study of the *Leuconostoc* genus which resulted in the introduction of the *Fructobacillus* novel genus. According to their analysis of 16S rRNA gene sequences, they sorted the *Leuconostoc* genus into three sub-clusters - the (1) *Leuconostoc mesenteroides* sub-cluster, the (2) *L*. *fructosum* sub-cluster and the (3) *L*. *fallax* sub-cluster (*L*. *fallax*). The *L*. *fructosum* sub-cluster consisted of *L. durionis, L. ficulneum, L. fructosum and L. pseudoficulneum*. This sub-cluster presented morphological and biochemical differences compared to the rest of the *Leuconostoc* species. The cells of the aforementioned species are all rod-shaped, they require an electron acceptor in order to ferment D-glucose, and the dissimilation of D-glucose in the suitable conditions yields acetic acid instead of ethanol. This led Endo and Okada to propose the novel genus *Fructobacillus*. As such, the four species were

also re-named accordingly; *Leuconostoc durionis* was modified to *Fructobacillus durionis* (Endo and Okada, 2008). *F. durionis* has been found to only play a minor role in specific fermentations, particularly of durian fruit, where it probably contributes to a desired sweet and sour final flavor.

The name of the *F*. *durionis* species is based on the durian fruit. Durian fruit is mainly grown in Malaysia, Indonesia, and Thailand and is most commonly consumed raw, cooked, or fermented into a condiment product known as "tempoyak" (Owens, 2014). Tempoyak is the source from which *Leuconostoc durionis* was initially isolated in 2005. Since then, it has only additionally been identified in Mexican coyol, *Acrocomia aculeate* palm sap wine and *Borassus akeassii* palm tree sap wines, and in cocoa bean fermentation (Leisner *et al.,* 2005; Alcantara-Hernandez *et al*., 2010; Papalexandratou *et al*., 2011; Ouoba *et al*., 2012). *F. durionis* was also isolated in the swabs and/or in the beginning of the first round of cocoa bean fermentation. However, it does not play a significant role as it is not isolated in later stages (Papalexandratou *et al*., 2011).

In addition, it was isolated from palm wines originating from Burkina Faso and Mexico (Alcantara-Hernandez *et al*., 2010; Ouoba *et al*., 2012). This may be unexpected given that this species had not been mentioned in wine studies before then. Ouoba et al. (2012) identified *F. durionis* in Bandji wine made from the fermentation of the sap of the *Borassus akeassii* palm tree. They aimed to study the most relevant micro-organisms which are "culturable". Among the thirty LAB that were identified, the *Fructobacillus* genus came in third place with 6.67% of total isolates whereas *Lactobacillus* was considerably the most predominant genus (86.67%). More specifically, *F. durionis* made up 6.67% of the total isolates (Alcantara-Hernandez *et al*., 2010 and Ouoba *et al*., 2012).

The exact roles played by the different species involved were not investigated as this was not the aim of the study. However, from what is known about *F. durionis,* its role can be deduced. *F. durionis* and *F. frusctosus* were only detected at the beginning of the experiment when their growth conditions were met (Alcantara-Hernandez *et al*., 2010). The growth requirements include aerobicity and the

presence of D-fructose or D-glucose with an electron acceptor. Once these conditions were no longer met, the species could not survive and disappeared. As with other FLAB, *F. durionis* and *F. fructosus* produce large amounts of mannitol from glucose and fructose fermentation which leads to the production of a sweet flavor and additional fermentation substrate for themselves. Like other LAB, they produce lactic acid, acetic acid, and traces of ethanol which contribute to the decrease in pH during fermentation of the sap which also influences the growth of other bacteria and imparts a sour flavor to the resulting product. The flavor of such wines as Bandji and taberna has simultaneous sweet and sour aspects that *F. durionis* probably contributes to (Ouoba *et al.,* 2012 and Owens, 2014). Like in the case of tempoyak, *F. durionis* can be used as an ingredient in fermentations of new beverages having a characteristic sweet and sour flavor.

All in all, considering the possible role of this species, there are limited options for future applications, especially given limited knowledge and its infrequent occurrence. While it is not likely that *F. durionis* will prove to be a candidate for probiotic or anti-pathogenic applications, its production of acid and mannitol may be regarder as some advantage (Endo and Okada, 2008, and Owens, 2014).

2.2.4. *Fructobacillus pseudoficulneus*

Leuconostoc pseudoficulneum was first isolated in 2006 and named due to it high sequence similarity to *Leuconostoc ficulneum* and to their similarities in isolation. Both species were isolated from ripe Portuguese figs, but were clearly distinct species. They differed with regards to their biochemical characteristics such as the results of the API ZYM microtubes, in chemotaxonomic properties, and according to DNA-DNA hybridization values (Chambel *et al.,* 2006). As previously mentioned, Endo and Okada performed a taxonomic study on the *Leuconostoc* genus. Along with the *Leuconostoc* species *fructosum*, *durionis*, and *ficulneum*, *Leuconostoc pseudoficulneum* was also found to belong to the novel *Fructobacillus* genus. It was thus renamed *Fructobacillus pseudoficulneus* (Endo and Okada, 2008). The main function of *F. pseudoficulneus* is that it produces high amounts of lactic acid and acetic acid from fructose and reconversion of mannitol into acid.

Additionally, it has been isolated from figs and bananas in South Africa and in small proportions in Mexican taberna sap wine (Endo *et al.,* 2009). It is also commonly isolated in cocoa beans, especially of Brazilian and Ghanaian origins, and occasionally in chocolate as a result of that (Alcantara-Hernandez *et al*., 2010; Armisen *et al*., 2010; Papalexandratou *et al*., 2011; Endo, 2012).

The role of *F. pseudoficulneus* in cocoa bean fermentation was used as the subject of a comparison between culture-dependent and culture-independent methods (Denaturing Gradient Gel Electrophopresis - DGGE). Results of DGGE generally agreed with those of the culturing method, but they revealed that the role of *F. pseudoficulneus* has been underestimated. The strength of the fingerprint bands provides better information about the influence of each studied species. DGGE also allows fermentation monitoring, which is advantagous over culture-based methods (Nielsen *et al*., 2006).

Subsequently to the study by Nielsen et al. (2006) other reports highlighted the role of *F. pseudoficulneus* in cocoa bean fermentation (Lefeber *et al*., 2010; Lefeber *et al.,* 2011). This means that the high fructose and citric acid level found in cocoa pulp provided ideal conditions for heterofermentative species like *F. pseudoficulneus*. Since these conditions are only available in the initial stage of the fermentation of the cocoa pulp, the prevalence and survival of decreases with their disappearance (Armisen *et al*., 2010; Papalexandratou *et al*., 2011). Moreover, it is probable that *F. pseudoficulneus* has a greater positive effect on the final product than was thought. Because it is an obligate FLAB, *F. pseudoficulneus* produces high amounts of lactic acid and acetic acid from fructose as well as mannitol (Chambel *et al*., 2006; Endo *et al*., 2008). This contributes alongside other yeasts and LAB to the decrease in pH. This low pH and high acid output contributes to desired results. The acidity contributes to the destruction of the pulp shell and to the promotion of flavor precursors and provides appropriate conditions for the development of colors, flavors, and other necessary processes (Camu *et al*., 2008; Lefeber *et al.,* 2011).

2.2.5. *Fructobacillus tropaeoli*

Fructobacillus tropaeoli was isolated from *Tropaeolum majus*, a nasturtium flower, in Stellenbosch, South Africa in June 2009 (Endo *et al*., 2011). Besides *Tropaeolum majus*, it was also identified in cocoa beans, papaya, figs, and Tempranillo red wine (Papalexandratou *et al.,* 2011; González-Arenzana *et al.,* 2012; Fessard *et al.,* 2017; Ruiz-Rodriguez *et al.,* 2017). *F. tropaeoli* grows on D-fructose faster than on D-glucose, and requires oxygen or another electron acceptor such as pyruvate to ferment D-glucose. Thus, it is a typical obligate FLAB and had closest sequence similarity to *F. ficulneus* and *F. pseudoficulneus* (Endo *et al*., 2011).

Soon after its isolation, a study by Papalexandratou et al. (2011) identified a species with a 98% similarity to *F. tropaeoli* in cocoa bean pulp mass. However, *F. tropaeoli* itself was later isolated from cocoa beans from Brazil, Ecuador, and Malaysia (Snauwaert *et al.,* 2013). Other FLAB isolated were *F. ficulneus* and *F. durionis* in the swabs and/or in the beginning of the first fermentation round. The presence of the *Fructobacillus* species and accompanying *Leuconostoc* species influences the fermentation process. Specifically, the spreading of the cocoa bean provides aerobic conditions for the aforementioned obligate FLAB to additionally ferment glucose. The microbiota is quite varied and includes acetic acid bacteria and yeasts. In traditional fermentation, the by-products of the bacteria negatively affect the chocolate product. The short fermentation time and the process of spreading the cocoa beans hinder proper flavor development and, thus, chocolate quality (Papalexandratou *et al*., 2011). Therefore, extending the duration of fermentation and avoiding the spreading of the cocoa bean would lead to an improved final product.

Later on, *F. tropaeoli* appeared in wine for the first time (González-Arenzana *et al.,* 2015). It was found in the same study which identified *F. ficulneus* in Tempranillo wine from the Rioja region in Spain. The appearance came in later years of the study when the aim was to compare the effectiveness of culture-independent methods to that of plating techniques. The results were convincing

when PCR-16S rDNA-DGGE identified both bacteria whereas culture-dependent methods did not (González-Arenzana *et al.,* 2015). Their isolation probably failed due to the unsuitability of the culture media and/or conditions.

Then in 2017, Fessard et al. isolated *F. tropaeoli* in papaya from the Reunion Islands. Their experiment found that it had one of the lowest growth rates on apple juice compared to the other strains used. Even though apple juice is considered a medium supportive of LAB growth, the amount of fructose and/or glucose it contains could have been too high to support the growth of *F. tropaeoli* (Fessard *et al.,* 2017).

In all previous studies, *F. tropaeoli* established itself as an effective producer of mannitol. Therefore, a study was performed on the mannitol producing ability of the strain *F. tropaeoli* CRL 2034. The stain was isolated from wild ripe figs in Tucumán, Northwestern Argentina. The strain produced one of the highest reported values of mannitol by LAB strains. After only 24hr of incubation, approximately 100g/L of mannitol were produced from a fructose/glucose mixture. The strain was grown under conditions that favored the easy isolation of pure mannitol. This strategy included subjecting the bacteria to osmotic and oxidative stress. Such undesired environmental conditions initiate mannitol production. Then the saccharide content of the medium used and the stirring speed could be adjusted to monitor for optimal production conditions. The results confirmed that *F. tropaeoli* CRL 2034 and secondary to it is *F. pseudoficulneus* (Ruiz-Rodriguez *et al.,* 2017). Therefore, the FLAB can be grouped with *F. fructosus* as having likely potential applications in industrial mannitol fermentation among other options (Carvalheiro *et al*., 2011).

3. Taxonomy

As previously mentioned, the FLAB consist of eight species belonging to two genera and two families under the order *Lactobacillales*. The genus *Lactobacillus* belongs to the family *Lactobacillaceae*, and the genus *Fructobacillus* belongs to the family *Leuconostocaceae.* The genus *Fructobacillus* was initially created based on noted similarities in morphological, biochemical, physiological, and, most importantly, phylogenetic criteria. *L. kunkeei* and *L. apinorum* were grouped into FLAB based on their similar biochemical characteristics to *Fructobacillus* species. They are also distinguished by whether they are obligate or facultative FLAB, *Lactobacillus florum* – even though only one bacterium counts as facultative so far.

3.1 Differentiating criteria of FLAB

FLAB are distinguishable from other bacteria by four main characteristics (Endo *et al.* 2009; Filannino *et al.* 2016).

- A preference for the fermentation of D-fructose instead of the typical LAB preferred substrate - D-glucose.
- FLAB produce carbon dioxide gas from glucose fermentation along with high amounts of lactic acid and acetic acid with trace amounts of ethanol. This means that they are not typical obligate heterofermenters.
- In general, FLAB show optimal growth when fermenting glucose under aerobic conditions or in the presence of an electron acceptor.
- A limited ability to ferment carbohydrates in general, and a notable inability to ferment starch, sucrose, galactose, mannose, amino sugars, and nucleotide sugars. The tolerance of different concentrations of sugar is strain dependent.

3.2 Facultative FLAB

This section highlights the differentiating properties of the facultative FLAB. The distinguishing features are clear, but the knowledge is limited to what is known

about the bacterium *L. florum* (Endo *et al*., 2012). The four main criteria for identifying a FLAB still apply, with two main modifications. First, the facultative FLAB have the ability to ferment D-glucose in the absence of electron acceptors (Endo *et al*., 2009; 2012). Second, the proportions of the end-products - ethanol, acetic acid, and lactic acid - may be substrate-dependent (Tyler *et al.,* 2016). It is worth noting that electron acceptors do improve the fermentation rate of D-glucose (Endo *et al*., 2009; 2010). Besides these two points, the facultative FLAB follow the trends of the obligate FLAB. *L. florum* possesses bifunctional alcohol/acetoaldehyde dehydrogenase gene (*adhE*), which is usually seen in obligately fermentative LAB but absent in obligately FLAB. The reason for fructophilic characteristics in *L. florum* is thus unclear.

3.3 Morphological characteristics

FLAB share a number of morphological traits. The cells are non-motile, Gram-stain positive, asporogenous rods. *Leuconostoc pseudoficulneum* was originally reported to possess coccoid-shaped cells, but the latter study clearly revealed that the species also possess rod-shaped cells. Tab. 2 shows additional morphological traits that, while not being identically shared, are highly similar. The noted identification media are the ones used during the initial isolation experiments (Edwards *et al*., 1998; Antunes *et al*., 2002; Leisner *et al*., 2005; Endo *et al*., 2006; Endo *et al*., 2010; Endo *et al*., 2011; Olofsson *et al*., 2014). It is worth noting that the cell morphology of the corresponding strains does not significantly vary with different culture conditions (Endo and Dicks, 2014).

3.4 Genomic evolution

Members of the *Fructobacillus* genus show similar patterns of enzymatic activity, and they are believed to have undergone regressive evolution in order to adapt to fructose-rich environments (Endo and Dicks, 2014). Evidence of such changes present themselves in the clear differences to otherwise close relative bacteria. The phenomenon is not a novel concept as niche-specific regressive evolution has been previously reported for LAB such as *Streptococcus thermophilus*

and *Lactobacillus delbrueckii* subsp. *bulgaricus.* The main function believed to have been lost is the *adhE* gene which is needed for the production of the bifunctional alcohol/acetaldehyde dehydrogenase enzymes which allow the organism to dissimilate glucose. Southern blot hybridization and gene specific primers have not picked up the *adhE* gene in *Fructobacillus* species. However, a function which has persisted is the use of fructose as both substrate and electron acceptor - this does not depend on the *adhE* gene (Endo and Dicks, 2014).

Tab. 2: Distinguishing morphological characteristics of FLAB (Endo and Dicks, 2014)

Species	Identification medium	Colony description	Colony size (mm)	Cell occurrence
L. kunkeei	MRS	Generally: concave, opaque; From wine: smooth, white	~1–2	Pairs and chains
L. florum	FYP agar, 30% FYP broth	Smooth; Beige	~ 1–2	Single, pairs, chains
L. apinorum	MRS agar (supplemented)	Round, smooth to rough surface; White, opaque	3–4	Single or pairs
F. fructosus	MRS, FYP	Small, smooth, round; Grey-white	Aerobic: ~1–2 Anaerob: ~0.1–0.2	Single or pairs
F. ficulneus	MRS, FYP	Smooth, convex; Grey-white	<1	Single or pairs
F. durionis	APT, FYP	Round, smooth; Off-white	<1–2	Single, pairs, chains
F. pseudoficulneus	MRS, FYP	Small, smooth, round, convex; Opaque, greyish-white	Aerobic: ~1–2 Anaerob: ~0.1–0.2	Single, pairs, chains
F. tropaeoli	GYP	Smooth; White	Aerobic: ~1–2 Anaerob: ~0.1–0.2	Single, pairs, chains

3.5 Phylogenetic relationships

FLAB differ phylogenetically from the rest of the LAB, and their strains form their own sub-cluster. Of the seven obligate FLAB species, two are *Lactobacillus*

species - *L. apinorum* and *L. kunkeei*. The remaining five species make up the *Fructobacillus* genus: *F. durionis*, *F. ficulneus*, *F. fructosus*, *F. pseudoficulneus*, and *F. tropaeoli* (Endo and Okada, 2008).

To start with, the five species were differentiated from the rest of the *Leuconostoc* genus. The new *Fructobacillus* genus was proposed according to results of sequencing of housekeeping genes using multilocus sequence analysis (MLSA). Sequences of gene intergenic spacer regions (ISR) alongside the 16S rRNA, *rpoC,* and *recA* genes were used to confirm that the genus *Fructobacillus* is phylogenetically distinct from *Leuconostoc* spp. (Endo & Okada, 2008). The range of similarity between 16S rRNA gene sequences are notably higher in comparison to the other *Leuconostoc*. Where the sequence similarity among *Fructobacillus* species is from 94.2% to 99.2%, only 90.4% to 94.4% is shared with the members of the *Leuconostoc* genus (Endo and Okada, 2014). Therefore, the comparison of 16S rRNA gene sequence similarity alone is not definitive. Regardless, the resulting phylogenies were consistent with results based on 16S rRNA. This means that analysis of ISR, *rpoC,* and *recA* gene sequences resulted in similar sub-clustering combinations as 16S rRNA (Endo and Okada, 2008). Again, 16S–23S rRNA gene ISR sequence similarity among *Fructobacillus* species ranged from 81.3 to 92.4%, while it only ranged from 69.2% to 80.1% similarity in comparison with the other *Leuconostoc* species (Endo and Okada, 2014). Subsequently, further sorting of the *Fructobacillus* genus based on 16S rRNA sequence similarity yields two sub-clusters: one consisting of *F. durionis* and *F. fructosus* and the other of *F. ficulneus, F. pseudoficulneus*, and *F. tropaeoli* (Endo and Dicks, 2014).

Next, the three species from the *Lactobacillus* genus – *L. kunkeei*, *L. florum*, and *L. apinorum* - were found to fulfill the criteria and were added to the FLAB group. When *L. kunkeei* was first isolated, the strain used was YH-15. After the determination of its almost complete gene sequence, the program FASTA of the Genetics Computer Group package was used to perform sequence searches of databases such as Genbank and Ribosomal Database Project libraries to identify the phylogenetically closest relatives. Its closest relatives were noted as species of the *Pedoicoccus* and *Lactobacillus* genera (Edwards *et al*., 1998).

Next, *L. florum* was characterized in 2010 as the first and only facultative FLAB (Endo *et al*., 2012). Its closest relatives are not FLAB; instead, *L. florum* is phylogenetically grouped alongside *Lactobacillus fructivorans, Lactobacillus homohiochii, Lactobacillus lindneri,* and *Lactobacillus sanfranciscensis*. However, the highest 16S rRNA gene sequence similarity is 95.4% with *L. lindneri* and 93.7% with *L. sanfranciscensi*s. Moreover, results of the DNA-DNA relatedness test on the aforementioned bacteria and the *L. florum* isolates ruled out potential relatedness. The other bacteria were not considered because the sequence similarities do not meet the cutoff value for species differentiation. Therefore, the tested *L. florum* strains were clearly considerably different even from their closest relatives. The results of 16S rRNA gene sequence similarity and levels of DNA–DNA relatedness see them clearly different from other species too (Endo *et al*., 2009; 2010; Endo and Okada, 2014).

Finally, the closest type strain to *Lactobacillus apinorum*, the most recently characterized FLAB, is *L. kunkeei*. In 2014, Olofsson et al. isolated the strain Fhon13N^T and found that it shared 98.9% 16S rRNA gene sequence similarity to *L. kunkeei*. In order to ensure that they are not strains of the same species, average nucleotide identity (ANI) analysis and DNA–DNA hybridization tests were performed. Results of the aforementioned tests found that the relatedness between Fhon13N^T and *L. kunkeei* were well below the recommended cut-off values. Later, the difference between Fhon13N^T and *L. kunkeei* was confirmed through the results of protein profiling tests performed using the MALDI-TOF MS. The conclusion of these tests is that *L. apinorum* and *L. kunkeei* are not the same species, but they are close relatives that are phylogenetically distinct from other *Lactobacilli* (Olofsson *et al.,* 2014). Fig. 1 below represents the species that make up the fructophilic LAB and shows their phylogenetic relationships. It is missing *Lactobacillus apinorum* which had not been isolated and characterized yet (Endo, 2012).

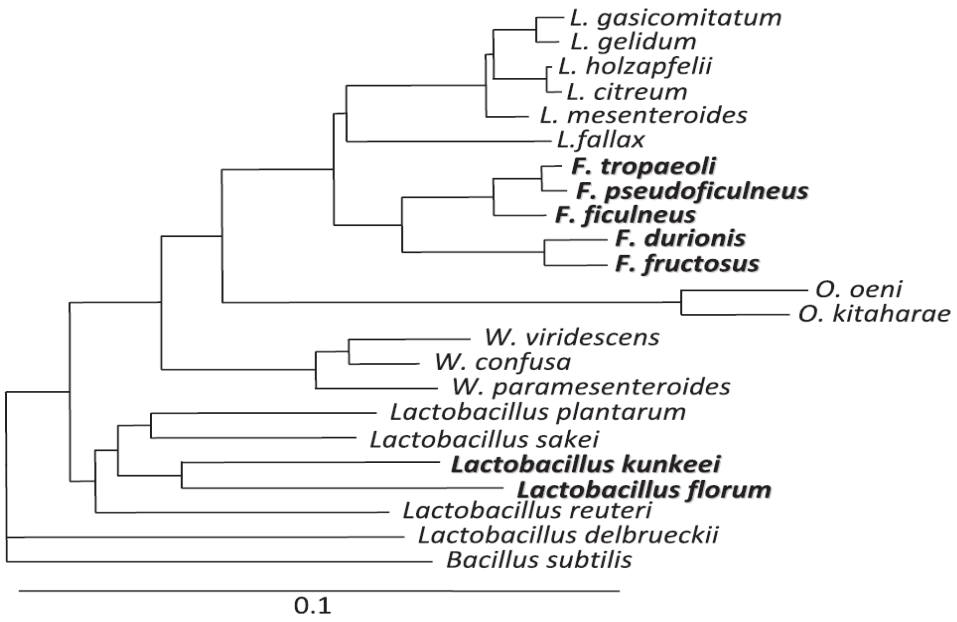

Fig. 2: Phylogenetically related LAB with highlighted species of Fructophilic LAB (Endo, 2012)

3.6 Physiological characteristics

It is not accepted to identify LAB by only considering physiological characteristics; phylogenetic and biochemical bases are required. However, once a bacterium is identified and its closest relatives are noted, physiological characteristics identify whether or not it is FLAB. The four main characteristics that serve as a sort of check-list in order to identify a species as obligate FLAB have already been listed in an introductory section (Endo *et al*., 2009). This sections serves to recapitulate and supplement what has been noted so far.

First and foremost, the bacteria must prefer to ferment D-fructose ahead of D-glucose. Second, the presence of an electron acceptor enables D-glucose fermentation. Third, FLAB grown on glucose-based media show enhanced growth under aerobic conditions as opposed to anaerobic ones. Finally, FLAB only have a limited ability to ferment carbohydrates in general, and are unable to ferment certain ones such as starch, sucrose, galactose, mannose, amino sugars, and nucleotide sugars (Endo *et al*., 2009). The products from the fermentation of glucose by obligate FLAB differ from other typical heterofermenters. The products themselves are the same, lactic acid, ethanol, acetic acid, and carbon dioxide. The difference is

that practically negligible amounts of ethanol are produced alongside approximately equimolar, high amounts of lactic acid and acetic acid. More specifically, the ratio of lactic acid:acetic acid is 1:1 for all *Fructobacillus* species, and it is 1:0.6-0.9 for *L. kunkeei* and *L. apinorum* (Endo and Okada, 2014; Maeno *et al*., 2017). Normally, almost equimolar amounts of lactic acid and ethanol are produced in heterofermentative LAB, and it is, instead, acetic acid production which is limited (Endo *et al*., 2012; Endo *et al*., 2014; Endo and Dicks, 2014). *L. kunkeei* produces D- and L-lactic acid at a ratio of 2:8. In contrast, members of the genus *Fructobacillus* produce D- and L-lactic acid at a ratio of 9:1 (Endo *et al*., 2011; 2012).

4. Biochemical properties and technofunctionality

4.1 Growth characteristics and cultivation

The growth of FLAB on fructose- and/or glucose-based media leads to different outcomes related to the ability of cultures to grow and the rate at which this takes place. There are also additional conditions that come into play such as pH, temperature, and aerobiosis. Tab. 3 displays different growth characteristics which are also related to culturing and the techno-functionality of the FLAB.

4.1.1 pH resistance

FLAB all grow within a common pH range, but each species additionally grows at other acidic or basic conditions. As can be seen in Tab. 3, all FLAB can grow between a pH of 5.0 and 7.0 in accordance with the acidic-leaning environments where they are found. *L. apinorum* was reported to grow through the most acidic and basic of conditions among FLAB (Olofsson *et al.,* 2014). The exact values require further validation as *Lactobacilli* are not known to grow above a pH of 10. *F. fructosus*, *F. ficulneus*, and *F. pseudoficulneus* grow optimally at nearly neutral pH. The pH tolerance of *F. durionis* has only been reported once upon its isolation by Leisner et al. in 2005. *L. florum*, *L. kunkeei*, and *F. tropaeoli* all grow

between 4.0 and 8.0 which shows a possible link to their microbiota origins (Endo and Dicks, 2014).

4.1.2 Temperature tolerance

Generally, it can be said that FLAB can grow between 15 and 35 °C. Temperature tolerance is, however, not accurately generalized upon for FLAB because the recorded values do not present a clear picture in half of the cases. In Tab. 3, the temperature ranges at which each FLAB is confirmed to grow are presented. However, there are uncertainties in the growth ranges of *L. florum*, *F. tropaeoli*, *F. ficulneus*, and *F. pseudoficulneus*. In the cases of *F. tropaeoli* and *L. florum*, only the optimal growth temperature was recorded at 30 °C and it was noted that growth could not occur at 45 °C – a lower limit was not noted. Similarly, *F. pseudoficulneus* can grow between 30 and 37 °C but not at 4 °C, but a higher limit was not noted. In the case of *F. ficulneus*, the optimal growth is again noted at 30 °C. But, it was noted that *F. ficulneus* can neither grow at temperatures as low as 6 °C nor as high as 40 °C - the cut off values are not clear. Of all FLAB, only *L. apinorum* can grow above 45 °C as it can tolerate up to 50 °C (Antunes *et al.,* 2002; Leisner *et al.,* 2005; Chambel *et al*., 2006; Endo *et al.,* 2010; 2011; Endo and Dicks, 2014; Olofsson *et al*., 2014).

4.1.3 Aerobiosis

Under anaerobic conditions, all FLAB grow better on fructose than on glucose. When fermenting glucose, the isolates also grow considerably better in the presence of 1% pyruvate or under aerobic conditions which may be created by shaking. In contrast, under aerobic conditions, FLAB seem to grow on glucose better than on fructose. Fructose fermentation on aerobic conditions requires further characterization to provide better evidence. FLAB seem to grow best when fermenting glucose under aerobic conditions. In this context, they grow second best in the presence of pyruvate. It is worth noting that the bacteria grow quite well when co-fermenting glucose and fructose. A likely explanation as to why glucose is the better fermented substrate under the right conditions is thought to be the difference

in the resulting ATP. Two moles of ATP are produced from 1 mole of glucose in the presence of oxygen. Similarly, 1.5 moles of ATP are produced from 1 mole of glucose in the presence of pyruvate. In contrast, only 0.67 moles of ATP are produced from 1 mole of fructose because it is utilized as both a substrate and an electron acceptor (Endo and Dicks, 2014).

Tab. 3: Select biochemical characteristics of FLAB (Endo and Dicks, 2014)

Species	Temperature tolerance	pH tolerance	Salt tolerance	Fructose tolerance
L. kunkeei	15 – 37 °C	3.7 – 8.0	5%	30% *
L. florum	15 °C **	4.0 – 8	5%	30%
L. apinorum	15 – 50 °C	3.0 – 12.0**	N/A	N/A
F. fructosus	6 – 40 °C	6.5 – 7.0	8.0%	50%*
F. ficulneus	30 °C **	6.5 –7.0	7.0%	40%
F. durionis	5 – 35 °C	3.9**	8.0%	40%
F. pseudoficulneus	30 °C **	4.8 – 8.5	3-6.5%	40%
F. tropaeoli	10 – 15 °C **	4.0 – 8.0	2.5%	30%

*Strain variations exist
**Has not been widely recorded
N/A Information is not available in literature

4.1.4 Osmotolerance

Almost all FLAB are osmotolerant, but they are not identical in their viability under different fructose, glucose, and/or salt concentrations. When it comes to media of high fructose concentrations, there is a limit above which growth is significantly reduced. *Fructobacillus tropaeoli* possesses the lowest fructose tolerance (Endo *et al*., 2009; 2011). It grows weakly at 30% (w/v) fructose - which is the lowest fructose concentration compared to the rest. All the other *Fructobacillus* species, however, do not show signs of hindered growth at 30% fructose. It is instead at 40% (w/v) fructose that they grow notably slower (Endo & Okada, 2008).

4.2 Efficiency of electron acceptors

When observing the growth rates on fructose and glucose, aerobiosis and added contributors showed drastic influence. FLAB grow well on fructose but it stood out that they grow best when fermenting glucose either in the presence of pyruvate, when fructose is also present, or under aerobic conditions. These scenarios have led to the conclusion that an electron acceptor is needed for enhanced fructose/glucose metabolism (Endo *et al*., 2009; 2011; 2012). It was also concluded that fructose itself is used as both a substrate and an electron acceptor. This lead to the investigation of the efficiency of different external electron acceptors. Results confirmed that the most efficient electron acceptors are fructose, pyruvic acid, and oxygen, but the effectiveness of different phenolic acids is strain-dependent (Endo *et al*., 2009; Filannino *et al.,* 2016). It was found that, of the phenolic acids, p-coumaric acid may be used as an electron acceptor, but not as efficiently as fructose and pyruvic acid (Filannino *et al*., 2016).

4.2.1 Enzyme activity

Most FLAB are catalase negative as can be seen in Tab. 4. In fact, only *L. florum* and *L. kunkeei* show some catalase positive activity in exceptional situations. Positive activity is seen when *L. florum* is cultivated on FYP agar containing sheep blood whereas *L. kunkeei* shows some positive activity in the presence of haem (Endo *et al.,* 2010; 2012). This suggests that these organisms possess heme-dependent catalase activity.

Biochemical properties and technofunctionality

Regardless of the fact that FLAB are obligate heterofermentative LAB, the difference in the yields of their products indicates differences at the biochemical level. The following observations are seen in the *Fructobacillus* genus as well as in the *L. kunkeei* and *L. apinorum* species, and they are clearly different from other *Lactobacilli* and from other LAB. Two explanations related to the corresponding enzymatic activity have been proposed so far. One is regarding the absence of the phosphoenolpyruvate-sugar phosphotransferase (PTS) system and the other is related to a deficiency in or the absence of the enzyme alcohol/acetaldehyde dehydrogenase and the *adhE* gene in members of the obligate FLAB (Endo and Okada, 2008; Endo *et al*., 2009; Endo *et al.*, 2012; Endo and Dicks, 2014).

Normally, the pentose phosphate pathway is used for glucose metabolism. The fermentation of pentoses is not possible by FLAB, and it is believed to be due to the absence of isomerase's and epimerase's. In LAB, it resulted in scarce amounts of acetic acid and ensured that ethanol is produced abundantly. However, in FLAB, weak ADH and no ALDH activity were detected, whereas NADH oxidase was highly active (Endo *et al*., 2014; Maeno *et al*., 2017). The *adhE* gene is currently believed to be absent in FLAB (Maeno *et al*., 2017). This, alongside the resulting fermentation products, supports the deduction that the alcohol/acetaldehyde dehydrogenase is either absent or dysfunctional in FLAB. Looking at the biochemical reactions leading to the increased production of acetic acid also clarifies the role of the electron acceptor. In order for acetic acid to be produced, the NAD(P)/NAD(P)H cycle must be balanced. This is achieved by the electron acceptor (Endo and Dicks, 2014). Heterofermentative LAB normally use acetyl phosphate as a substrate to produce ethanol and regenerate NAD+ via acetaldehyde. In the case of FLAB, looking backwards through the reaction, acetyl phosphate would be used to produce acetic acid via acetate kinase (Koo *et al*., 2005). This reaction alone would leave a deficiency in NAD+. The electron acceptor, therefore, becomes crucial to maintain the balance of the NAD(P)/NAD(P)H cycle.

In addition, the phosphotransferase system (PTS) as well as the ubiquinone and other terpenoid-quinone biosynthesis are absent in FLAB (Maeno *et al*., 2017).

In cases where fructose is not the only carbon source under aerobic conditions, fructose is weakly fermented, it is likely to be mainly used by FLAB as an electron acceptor rather than as a substrate. This indicates that the activity of the corresponding fructose-PTS which is a fructose/mannitol-specific transporter or another phosphoenolpyruvate-sugar phosphotransferase (sugar PTS) of fructose is weak (Endo & Okada, 2008; Endo *et al*., 2009; Endo *et al.*, 2012; Endo and Okada, 2014; Maeno *et al*., 2017).

4.2.2 Bioactive metabolites

The existing knowledge about the metabolites produced by FLAB has only recently been published only for *L. kunkeei* and *L. apinorum* strains residing in the guts of honeybees (Olofsson *et al.,* 2016a). The production of organic acids, free fatty acids (3-OH FAs), 2-heptone, volatiles, and biofilm was detected upon cultivation with known human and bee pathogens (Olofsson *et al.,* 2016a). The role played by each of these metabolites can be found in Tab. 10 in Appendix II (see page 84). Results showed that the organic acids L-lactic acid, formic acid, and acetic acid were produced in varying amounts by all tested strains (Olofsson *et al.,* 2016a; Piccart *et al.,* 2016). In terms of free fatty acids, *L. kunkeei* Fhon2 and *L. apinorum* Fhon13 contained the highest amount. The 3-OH FAs were C 10:0, C 12:0, C 14:0, C 16:0, and C16:1. *L. kunkeei* Fhon2 and *L. apinorum* Fhon13 also produced the toxic volatiles toluene, xylene, and ethylbenzene. Specifically, strain Fhon2 produced the most xylene whereas Fhon13 was the main producer of ethylbenzene and produced a small amount of nonane. Notable, again, was the ability of Fhon13 to produce the highest amount of 2-heptanone among the 13 strains studied. The amounts of bioactive metabolites recorded by Olofsson et al. can be found in Tab. 8 (see page 83). Finally, biofilm formation was successful *in vitro*, but the strains Fhon2 and Fhon13 have a relatively weak ability to form biofilms (Olofsson *et al.,* 2016a; Silva *et al*., 2017). The metabolites produced by *Fructobacillus* FLAB have not been closely studied yet.

4.2.3 Carbohydrate fermentation

Another differentiating characteristic of FLAB is that they only ferment a small number of carbohydrates (Endo and Dicks, 2014). However, carbohydrate fermentation cannot be used as a differentiating criterion among FLAB as there is no clear trend. The only observations that can be generalized is the universal fermentation of fructose, glucose, and mannitol and the inability to ferment pentoses. As has previously been mentioned, fructose is fermented at the fastest rate, followed by glucose, and mannitol fermentation is the most delayed. The fermentation of additional carbohydrates is also observed by non-*Fructobacillus* FLAB as can be seen in the Tab.4 below. The predicted metabolic pathway of mannitol production from FLAB fermentation can be found in Fig 3. in Appendix I (see page 80).

Tab. 4: Additional biochemical characteristics of FLAB

Species	Catalase	G+C content of DNA	Carbohydrate fermentation
L. kunkeei	Positive**	36.0 mol%	D-Fructose, D-glucose, D-mannitol, sucrose
L. florum	Negative[1]	42.0 mol%	D-Fructose, D-glucose
L. apinorum	Negative	34.7 mol%	D-Fructose, D-glucose
F. fructosus	Negative	43.4 mol%	D-Fructose, D-glucose, D-mannitol
F. ficulneus	Positive	42.6 mol%	D-Fructose, D-glucose, D-mannitol, sucrose, D-turanose, gluconate*, maltose*, trehalose, methyl-α-D-glucopyranoside*
F. durionis	Negative	44.0 mol%	D-Fructose, D-glucose, D-mannitol, sucrose, D-turanose, D-ribose*, gluconate*, maltose, trehalose, methyl-α-D-glucopyranoside
F. pseudoficul.	Negative	44.5 mol%	D-Fructose, D-glucose, D-mannitol
F. tropaeoli	Negative	44.0 mol%	D-Fructose, D-glucose, D-mannitol, D-turanose [2], gluconate **

[1] Negative unless with sheep blood
[2] Variable

* weak
** weak or not at all

5. Safety assessment of FLAB

Even though there is growing evidence of the beneficial properties of FLAB, they will have to undergo the established evaluation processes if they are to be used in food and feed. The precautionary approach of the EU is upheld in the interest of protecting the consumer. It seems to be plausible that FLAB will not take too long to be authorized as novel food cultures and further be included in the QPS list (Brodman *et al.,* 2017).

5.1 Legal status and potential for approval

The required approval of FLAB as novel foods by the EU, the recognition as GRAS by the USA, and addition to the QPS list and other food safety tools are the last obstacle. In the EU, novelty has been defined with reference to a set deadline. The definition states clearly that food and food ingredients that consist of microorganisms fall under its authority. Any food that was not significantly consumed prior to the 14th of May 1997 has to comply with the Novel Food regulation EU 2015/2283 (see Appendix I page 81). Since the recent discovery of FLAB and taking into account that they hitherto have not been consumed in significant amounts, they are considered novel foods by the EU. An exception could possibly be made related to *L. kunkeei and L. apinorum* considering the traditional consumption of honey even though they are not among the dominant microbiota.

The introduction of a novel food to consumers is subject to a pre-market evaluation and authorization procedure. EFSA performs a risk assessment and the EU Commission performs a risk management (Laulund *et al.*, 2017). The first step is create tangible products that incorporate the different FLAB and LAB mixtures. These should then be evaluated for possible counter reactions or reduced efficiency in light of being present in novel matrices and stresses. Finally, the durability and impact on the consumer's gut microbiota should be evaluated rigorously for short and long term exposure (Laulund *et al.,* 2017).

In order to be approved, the different strains have to continuously show no cases of sepsis, endocarditis, or bacteremia like certain strains of *Lactobacillus* have. They should not exhibit virulence such as hemolytic or cytotoxic activity in *in vitro* studies (Carasi, 2014; Laulund *et al.,* 2017). In terms of toxicity, no deleterious effect was found for FLAB in *in vivo* models and this scenario was also predominant in *in vitro* trials (Arrendodo *et al.,* 2017). They also should not exhibit an acquired antimicrobial resistance – this is essential for starter cultures and probiotics. *F. fructosus* and *L. kunkeei* have exhibited differing levels of susceptibility to paratransgenesis (Maddaloni *et al.,* 2014). However, this is not cause for concern regarding antibiotic resistance knowing that antibiotic resistance genes cannot be transferred to non-pahogenic microorganisms (Laulund *et al.,* 2017). The EFSA has already defined breakpoints for heterofermentative lactobacilli regarding minimum inhibition concentrations for a list of antibiotics (Carasi, 2014). EFSA accept or reject the strain based on the results of antibiotic susceptibility tests (Carasi, 2014; Laulund *et al.,* 2017).

5.2 Safety assessment toolbox

Currently, the toolbox for the assessment of strain safety comprises several components. These tools include the QPS List by EFSA and the Inventory of Microorganisms with Technological Beneficial Use by the EFFCA and of the IDF. Additional tools include phenotypic methods in which novel applications have been introduced aiming to improve the assessment of the safety of food cultures. Phenotypic methods include molecular and nucleic acid-based methods, second-generation sequencing technologies, and Whole Genome Sequencing (WGS). WGS is recommended to replace conventional genetic fingerprinting techniques like Pulse Field Gel Electrophoresis (PFGE) and surpass it with additional insights through analyzing the genetic bases of strains. The assessment of WGS can be done using methodologies such as the MvirDb database of microbial virulence factors, the CARD database, the ResFinder and VirulenceFinder databases. These tools facilitate the identification of both antibiotic resistance and virulence genes that are acquired by phage genomes. Besides assessing the genome, biogenic amine (BA)

production must be assessed through an Induction test. BA's are undesired substances which include histamine, tyramine, putrescine, phenylethylamine, and cadaverine. A screening of genes involved in BA formation should also be performed. The procedure of the induction test is simple as it includes the adding the precursors of the amino acids in question and leaving the strain to grow (Laulund *et al.,* 2017). In terms of risk assessment, the FLAB so far have not been linked to any hazards and exposure assessments in different studies have not reported any adverse effects related to dosage.

5.3 Safety criteria

There is no evidence linking FLAB to any pathogenic properties. However, the safety of novel microorganisms should not be taken lightly because neither the exposure of consumers to them is known, nor are the long term effects of increased doses with the food matrix or human and animal body (Brodman *et al.,* 2017). The general attitude towards FLAB is currently positive and optimistic.

One of the obstacles to this is the risk posed by virulent bacteriophages as these can ruin the fermentation and/or retard food processing (Giraffa, 2014). As of yet, there are no bacteriophages reported for the *Fructobacillus* genus, but some are found for *Lactobacillus* (Kot *et al*., 2014; Mercanti *et al*., 2016). This presents some cause for attention because it is believed that high species relatedness may be a facilitator of phage development. Namely, prophages exist for *L. sanfranciscensis* which means that special attention should be paid to its close phylogenetic relative *L. florum* (Endo *et al*., 2010; Mercanti *et al*., 2016). There has been a great development in phage detection and genomics over the past decade. They are usually encountered in strains that are used regularly as fermentation cultures – mainly for dairy products (Mahony *et al.,* 2014; Mercanti *et al*., 2016). As a number of FLAB might have future applications as starter cultures, this may be an area worth monitoring. Of course, basic practices must be performed such as ensuring the appropriate storage temperature, tracing ability, accurate documentation, and avoiding reaching a high number of generations from the reference stock. Additionally, the genetic stability of the strains in questions must be closely

monitored and documented. The use of WGS or PFGE fingerprinting can be used to ensure that no genetic drift is occurring. Standard quality control testing should include a comparison of the genomic profile and plasmid content of the inoculation strain to the strain's reference stock material (Laulund *et al.,* 2017).

In addition, many FLAB strains exhibit antimicrobial or probiotic activity, which is highly desirable in modern clinical settings. Usually, probiotics used have a reputation of safe use over long periods of time and they would be species and strains that already exist in the GI tract of the target host. As FLAB are a novel group and have not been detected in vertebrates' GI yet, they require further evaluation (Endo and Salminen, 2013). What is encouraging is that they have all been consumed one way or another in small amounts across history, and that they are continuously showing positive results against different pathogens. The safety concern caused by the non-detection of FLAB in human guts may be alleviated by a process of heat treatment. This observation was initially pointed out by Plovier and colleagues as they found that *Akkermansia* cells, rendered non-replicating, retained the ability to provide beneficial effects. This will still, of course, require extensive trials and close monitoring, but human and animal trials have shown positive results (Asama *et al*., 2015; 2016).

Finally, the implementation of the safety regulations regarding food cultures may differ from country to country, although there is one single set of rules within the EU. Nevertheless, the need to evaluate the safety of relevant microorganisms at strain level, during production, throughout the shelf life of the food item, and its effects after consumption is mandatory (Laulund *et al.,* 2017).

6. Applications of FLAB

The natural food sources of FLAB and their biochemical and physiological characteristics are indicative of potential food and health purposes. As the applications of LAB in food are wide-ranging, there is a number of opportunities for fructophilic LAB to be taken advantage of. From what is known so far, the applications can be classified into three categories: food product development, health promotion, and chemical production. FLAB do not share the same biofunctional properties. Therefore, some species may only play secondary roles in investigated applications. Tab. 5 briefly summarizes potential applications of FLAB. Further details can be found in Appendix I (see pages 70-80).

Tab. 5: Summary of potential applications of FLAB

Potential application	Relevant FLAB	Focus	Limitations	Citations
Probiotic benefits	*L. kunkeei, L. apinorum, F. fructosus*	Improvement of bee, animal, and human health	More research and approvals required	Asama *et al.*, 2015; 2016;
Antagonistic properties	*L. kunkeei, L. apinorum*	Treating animal and human pathogens with solutions from bee products	FLAB only grow in honey < 2 weeks old. More research and approvals required	Olofsson *et al.*, 2016a and b; Butler *et al.*, 2016; Mårtensson *et al.*, 2017
Production of lactic acid	All FLAB	Use in food production and pharmaceuticals	Expensive method	Vijayakumar *et al.*, 2008
Production of mannitol	*F. tropaeoli, F. fructosus, F.pseudoficulneus*	Use as a natural sweetener, osmotic diuretic	Not cost-effective	Tyler *et al.*, 2016; Rodriguez *et al.*, 2017
Production of erythritol	*F. tropaeoli, L. florum*	Use as a natural sweetener	Not cost-effective	Tyler *et al.*, 2016 Rodriguez *et al.*, 2017
Starter cultures	All FLAB except *L. apinorum*	Product improvement: beverages and condiments.	More research and approvals required	Amiza *et al.*, 2006; Yuliana and Garcia, 2009 Owens, 2014
Functional cultures	All FLAB except *L. apinorum*	Process optimization, product development	More research and approvals required	Alcantara-Hernandez *et al.*, 2010 Papalexandratou *et al.*, 2011 Ouoba *et al.*, 2012

7. Final comments on limitations of current literature

Given the novelty of the group's characterization, there are, conceivably, quite a few gaps in knowledge at the level of the literature. The literature has been slightly hindered by non-identical documentation of bacterial features, experimental errors, and inappropriate methodology. Moreover, further tracing of the evolution of each species will help predict future adaptations.

First, several studies do not conform to a standardized methodology and language. Thus, they have not always presented a uniform record of tested criteria. For example, the absence of gas production by *L. ficulneum* was not reported by Antunes et al. (2002) so it could not be compared to *L. durionis* and *L. fructosum* until later papers (Leisner *et al.,* 2005). Similarly, the biochemical characterization of *L. kunkeei* species was not properly documented in the original article reported by Edwards et al. (Edwards *et al*., 1998). While the previous two cases have been sorted, the range of temperatures that support growth and pH and tolerance of salt and fructose have not been accurately defined for all FLAB as can be seen in Tab. 3. Furthermore, different studies have focused on the bacteria residing in the guts of honeybee larvae without differentiating their exact developmental stage. This is significant because the physiological changes in each preimaginal stage are highly dynamic and can, therefore, influence results (Hroncova *et al*., 2015). On a different note, the members of the FLAB have not all been treated equally with regards to finding relevant applications. *F. pseudoficulneus* has been described as the most commonly encountered FLAB in nature, but no significant attempts have been made to utilize it.

Second, experimental errors and inaccuracies are regularly come across. The numbers of FLAB present in samples are probably underestimated if the media used in identification only contains glucose as the source of fermentable carbohydrates (Leisner *et al*., 2001). Endo et al. (2008) proposed the use of media with glucose and fructose when isolating bacteria from fructose rich sources to avoid such errors. Also their heterofermentative metabolism may be in avertedly missed if

the usual gas-detecting media with glucose are used (Owens, 2014). Also, the isolation of *F. fructosus* from Spanish blood sausage and Zimbabwean wild fruit by Santos et al. in 2005 and Nyanga et al. in 2007 is likely to be a result of identification error. Neither were the culturing medium and conditions nor the differentiation criteria optimal for FLAB identification (Endo and Dicks, 2014). Similarly, the filters on the electrophoretic gel that was used to identify the proteins and peptides in honey by Olofsson et al. (2016a) were not large enough to allow the migration of bigger-sized proteins, so the results were altered accordingly.

Third, FLAB significantly began appearing among the results of studies after culture-independent methods became more popular. In fact, culture-independent methods enabled the detection of bacteria that plating analyses did not pick up (González-Arenzana *et al.,* 2012). The aforementioned culturing bias was justified as it was only rarely that culture-dependent methods detected more LAB species (Hyun *et al.,* 2014; González-Arenzana *et al*., 2017). The analysis of the 16S rRNA gene and novel technologies such as MALDI-TOF MS have shown that microbial populations are significantly more diverse than was previously thought and that dominant microorganisms may mask other bacteria (Hyun *et al.,* 2014). That said, questions have been raised about whether their presence has been overestimated on culture-independent methods (De Bruyne *et al.,* 2011).

Finally, the major gaps in knowledge concern the identification, characterization, and selection of the different existing FLAB strains, and to attempt to perform a dose-response assessment for prolonged exposure. What is left is to acquire a greater understanding of the FLAB strains' existing resistances and sensitivities, a registry of the amounts of bioactive metabolites they produce, and an investigation of possible toxicities or allergenicity (El-Ghaish *et al.,* 2011).

8. Outlook

Modern molecular and genome sequencing techniques have been divisive in their contributions to our knowledge of FLAB, and the developments in this field suggest even further breakthroughs. For example, as the detection of bee pathogens is increasingly necessary, metabarcoding analysis of the 16S rRNA gene has shown promise in this regard (Erban *et al.,* 2017). Moreover, strains that are probably weakly pathogenic are believed to differ because of geographical and temporal criteria according to 16S rRNA analyses (Erban *et al.,* 2017). Additionally, modern molecular techniques can be used to observe microbial and chemical changes to be used in process optimizations and modifications (Owens, 2014).

That said, certain techniques exist which are more controversial than other – namely paratransgenesis and other metabolic engineering. Molecular engineering aims to modify genomic components such as proteinases, peptidases, aminotransferases, enzymes for amino acid biosynthesis, and transport systems for peptides and amino acids in order to create more desirable characteristics. This can lessen the need to find novel strains and improve the efficiency of already used strains (Giraffa, 2014). The investigation of *F. fructosus* in paratransgenesis studies may lead to improved honeybee colony health by improving its probiotic properties, production of bioactive peptide and other metabolites, and by affording it the ability to dissimilate galactose as a carbohydrate source. This would allow the use of milk in honeybee feed.

The disadvantage is that GMM's and GMO's are not generally supported in the EU and many other parts of the world. Another limitation of paratransgenesis is the risk of negatively impacting the host's health – which is a highly beneficial creature in the case of FLAB (Maddaloni *et al.,* 2014).

9. Summary

The Fructophilic Lactic Acid Bacteria (FLAB) are a sub-group of LAB that was initially created based on noted similarities in morphological, biochemical, physiological, and phylogenetic criteria. The main distinguishing feature of FLAB is the preferential fermentation of D-fructose. As such, FLAB can be isolated from fructose-rich sources such as the gastrointestinal tracts of honeybees, as well as bee products, multiple flowers, and select ripe fruits and wines.

In nature, members of the FLAB are involved in varying degrees in developing certain organoleptic properties of food products by producing useful polyols, and contributing to improved host health through probiosis and releasing bioactive metabolites. FLAB species are quite similar in colony appearance, size, and cell occurrence. They differ, however, in growth-promoting temperatures and pH, and in viability under different fructose, glucose, and/or salt concentrations. Aerobiosis as a determinant of fructose and glucose fermentations has been investigated but aerobic fermentations require further elucidation.

In the EU, FLAB are considered as novel foods under regulation (EU) 2015/2283. Their introduction to consumers is thus subject to a pre-market evaluation and authorization procedure. In this regard, the encouraging results of trials on humans, animals, and insects suggest that FLAB are likely to be authorized as novel food cultures and included in the QPS list.

As can be expected, gaps in knowledge exist. The literature has been somewhat hindered by non-identical documentation of bacterial features, experimental errors, and unsuitable methodology. The main limitation, however, is that further research is needed to ensure safety at strain level, during processing, storage, throughout the shelf-life, and on host health post-consumption or application. The development of scientific opinions regarding FLAB may be related to advances in and applications of modern molecular and genome sequencing techniques.

10. List of tables

11. List of figures

12. Bibliography

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13. Appendix I

The appendix I contains additional relevant information that helps give a more comprehensive understanding of FLAB identity and applications. It, therefore, includes some marginal details about *L*. *kunkeei*, an overview of a fructophilic bacterial strain that may become recognized as FLAB in the future, and an elaboration of the fields in which FLAB can be utilized. The applications are discussed here in terms of their relevance, the mode of action, and their chances of success.

Lactobacillus kunkeei

Back when the first strain was being isolated and studied in 1996 and in 1998, it was clear that *L. kunkeei* was a unique, novel species. The consensus was to classify it as a *Lactobacillus* after finding that it did not fit in the genera *Pediococcus* or *Leuconostoc*. It was not similar enough to any of its closest relatives as shown by >7 % sequence divergence values based on 16S rRNA gene sequences (Edwards *et al.,* 1998). Even after the conception of the *Fructobacillus* genus in 2008, *L. kunkeei* was still classified as a species in genus *Lactobacillus.* However, it became considered an obligate FLAB in 2009 (Endo *et al.,* 2008; Endo *et al.,* 2009). Moreover, the description was emended in 2012 when Endo et al. performed the first isolation of *L. kunkeei* from wine since Edwards et al. in 1998. There were a number of strain-dependent characteristics, but the type strain fit the criteria of an obligate FLAB. Besides in wine, different strains of L. kunkeei were isolated from different sources in other countries seemingly all over the world.

To start with, Endo et al. (2009; 2012) performed numerous studies on *L. kunkeei* in South Africa when aiming to further characterize the species as part of the FLAB. As part of this, *L. kunkeei* was isolated from South African samples of Azalea, Cosmos, Nacissus and Japanese samples of Crape myrtle and Morning glory flowers. Additionally, it was isolated from Japanese honey, American red wine, and from local South African wines (Endo *et al*., 2009; Endo *et al.,* 2012). Moreover,

in Panama, McFrederick et al. (2014) isolated *L. kunkeei* in bee pollen and the digestive tracts of two species of halictid bees.

In addition, Anderson et al. (2013) took samples from honeybees' food which is stored in the hive and from the honeybees' digestive tract. The analyzed stored food included honey and beebread. Anderson et al. (2013) aimed to confirm the identity of the core bacterial species that dominate the guts of honeybees. The studies took place in Tucson, Arizona, USA. Samples were taken from nectar from the flowers that were in the immediate vicinity. Also, samples were taken from the bee alimentary tract and from the hive food. Results identified *L. kunkeei* strain Fhon2 in high amounts in the crop and hindgut. To a lesser degree, *L. kunkeei* was found in the midgut, floral nectar, beebread, and in pure honey. Additionally, it was noted that *L. kunkeei* was ever-present regardless of seasonal change, media usage, and aerobicity during incubation. It grew on MRS, BHI, and SDA media under both aerobic and microaerophilic conditions (Vásquez *et al*., 2012; Anderson *et al.,* 2013).

Observed variations in experimental results may well be due to geographical factors, mainly the weather difference between the cold of Scandinavia and heat of Southern USA, for example. Also, the observed variation is probably due to two main factors. The first factor is the nature of social insects such as honeybees. The changes in the environment prompt individual bees to adapt their collection, utilization, and consumption of nutrients accordingly. This is why, during the colder months, around 10% of the colony hibernates, while the rest strictly limit their consumption of honey (Tamarit *et al.,* 2015). And more, overwintering worker bees keep stored glycolipoproteins in their abdomens to provide to the developing larvae during the spring (Anderson *et al.,* 2014). Furthermore, fructose abundant in nectar is ingested during the warmer months. This provides the main growth substrate of *L. kunkeei* (Tamarit *et al.,* 2015).

Appendix

Lactobacillus fructivorans

L. fructivorans is not a member of the FLAB, however, a novel strain has shown FLAB properties which may indicate environmental adaptations (Li-Oon Chuah *et al*., 2016). It was isolated from spoiled salad dressing by Charlton et al. in 1934. It is usually encountered as a spoilage LAB that is also involved in generating particular flavors and ripening fermented food (Bjorkroth and Korkeala, 1997; Nam *et al*., 2012). A strain has been identified in tempoyak for the first time by Li-Oon Chuah et al. (2016) and it was considered FLAB for exhibiting fructophilic properties. First, it shares morphological similarities to FLAB as cells are rod-shaped, non-motile, non-spore-forming cells. Second, they are usually Gram positive, catalase negative, facultative anaerobes (Dicks *et al*., 2009; Konig and Fröhlich, 2009). Moreover, it is heterofermentative and grows on similar media as FLAB; i.e. MRS and FYP broth and ag96ar which contain fructose (Bjorkroth and Korkeala, 1997; Endo, 2012; Li-Oon Chuah *et al*., 2016). It also grew poorly in GYP unless if supplemented with D-fructose or pyruvate. This, however, is a characteristic of LAB in general, and not specific to FLAB (Endo *et al.,* 2009). As FLAB usually ferment only a few carbohydrates, the fact that it only fermented glucose and fructose is fitting (Li-Oon Chuah *et al*., 2016). In order to differentiate LAB from FLAB, aerobic culturing should be performed. The rate of fermentation of glucose to that of fructose should be compared. The growth in 30% fructose environments is another characteristic (Endo *et al.,* 2009). It should be differentiated whether *L. fructivorans* is obligate or facultative heterofermentative. The production of large amounts of lactic acid and acetic acid from D-glucose is indicative of obligate FLAB. The production of lactic acid, acetic acid, and ethanol in the ratio 1:0.2:0.8 corresponds to facultative, heterofermentative FLAB; both cases are different from other LAB (Endo *et al.,* 2009). As it is closely related to *L. florum*, the only facultative FLAB, it may be a second such case. It may be that this strain has undergone the same regressive evolution as other FLAB have in order to adapt to the high fructose environment. However, further phylogenetic analysis and biochemical confirmation tests are required before declaring the strain of *L. fructivorans* a stand-alone species or a strain which is part of the FLAB (Mtshali *et al.,* 2012).

Appendix

Applications

The different application categories will be elaborated here below; they will be discussed in order of relevance to today's societies starting with health promotion, followed by food product improvement, and ending with the more efficient production of different chemicals. New possibilities can be based on the physiological and biochemical interactions of the bacteria with its surrounding environment. This leads to a greater understanding of the mechanisms behind flavor formation, carbohydrate metabolism, adaptability, and stress responses (Giraffa, 2014). It equally facilitates the creation of a context to understand disease resistance and climatic adaptations (Wallberg *et al.*, 2014).

Health promotion

Probiosis

FLAB associated with potential probiotic features are *L. kunkeei, L. apinorum,* and *F. fructosus*. The fact that bacteria are already clearly present in the human and animal digestive tract in principle supports the perception of safety of the bacteria. Unfortunately, FLAB have not been detected in vertebrate digestive tracts (Endo and Salminen, 2013). Instead, they are quite commonly isolated in different parts of bee GI, bee products, and in guts of other high fructose-consuming insects. In this respect, it is quite straightforward to consider FLAB as insect probiotics, especially given that *L. kunkeei* is considered one of the most important probiotic producers of bacteriocins (Silva *et al*., 2017).

Among the different members of FLAB, *L. kunkeei* seems to be the most relevant species with health-promoting activities (Asama *et al*., 2015; 2016). In a study dealing with the effect of heat-treated *L. kunkeei* on the immunity, the dosage was taken as a 1000 mg pill per day over 4 weeks (Asama *et al*., 2015). The exact role and efficiency of the YB38 and YB83 isolates should be further investigated *in vitro* and, eventually, *in vivo*.

Appendix

In the second study, the *Lactobacillus kunkeei* YB38 strain was again heat-killed and administered at different doses over two weeks each in a human trial. The benefit of this study is that it showed the effect of different dosages on the human gut (Asama *et al.,* 2016). The dosages should be adapted according to the different age, species, dietary habits, current health status of the target population, and the desired effect in future studies.

Regarding the role of prebiotics in stimulating the growth of probiotics, a possible limitation is the fact that FLAB usually only ferment a limited number of sugars. In such a context, prebiotics naturally found in environments where FLAB normally grow and survive may be more likely to succeed. For example, malto-oligosaccharides, isomaltose, cellobiose, panose, maltotriose, melezitose, raffinose, maltose, turanose, and maltotriose are such prebiotics and also naturally found in honey (Pranckutė *et al.,* 2016; Silva *et al.*, 2017). The intention to promote the health of wild bees is an interesting approach. Therefore, field studies are currently underway to assess the effect of *L. kunkeei* on wild colonies (Arredondo *et al.,* 2017).

As a result of the knowledge collected so far, proposed applications include effective mixtures of the relevant *Lactobacillus* and *Bifidobacterium* strains which may also be tailored to the GI tract of the target organism. It is known that different strains of bacteria such as *L. kunkeei, L. helsingborgensis,* and possibly *L. apinorum* and *F. fructosus* are relevant candidates because they synergistically contribute to honeybees' health maintenance (Silva *et al.,* 2017). However, humans, bees, and animals are likely to respond differently due to their natural differences in gut microbiota (Silva *et al.,* 2017). Besides their biofunctionality, the beneficial bacteria need to be stable during processing and storage, and later on during gastrointestinal passage (Levin, 2011). Hence, strain selection based on stability and tolerance testing are essential.

Appendix

Antagonistic properties

Olofsson and Vásquez, had isolated microbiota from the guts of honeybees existing all over the world (2008). A more recent study on the bacterial metabolites responsible for maintaining bee immunity was described with reference to *L. kunkeei* and *L. apinorum* strains (Olofsson *et al.,* 2016a). Organic acids, free fatty acids (3-OH FAs), hydrogen peroxide, 2-heptone, volatiles, and biofilm formation are now recognized as the sources behind the antimicrobial properties. However, the detected proteins and peptides were even traced to non-FLAB bacteria. Therefore, more research is needed to identify them and understand their properties (Olofsson *et al.,* 2016a; Silva *et al*., 2017). Encouraged by the results of this study, this same set of bacteria is being tested as a means against known human and animal diseases, wounds, and *in vitro* on known pathogens. A spray, cream, or honey replica gel can be produced and used for its antimicrobial effect as a topical antibiotic. The success of such an endeavor would have a generally positive impact on human, animal, and bee lives, the world economy, and the environment.

The relevant FLAB strains and the role of the different metabolites will be elaborated here based on the results of the study by Olofsson *et al.,* (2016a). The eight strains identified as *Lactobacillus* were Bma5, Hma2, Hon2, Bin4, Hma8, Biut2, Fhon2, and Fhon13. Fhon2 and Fhon13 correspond to *L. kunkeei* and *L. apinorum* respectively. The five strains identified as *Bifidobacterium* were as Bin7, Bin2, Hma3 and Bma6. The focus of the study was to detect and identify the different metabolites produced by each strain. Results showed that the organic acids L-lactic acid, formic acid, and acetic acid were produced in varying amounts by all tested strains. These lower the pH of the environment making it difficult for other bacteria to survive (Olofsson *et al.,* 2016a; Piccart *et al.,* 2016). In terms of free fatty acids, *L. kunkeei* Fhon2 and *L. apinorum* Fhon13 contained the highest amount. The 3-OH FAs were C 10:0, C 12:0, C 14:0, C 16:0, and C16:1. The 3-OH FAs are relevant because of their ability to kill or inhibit the growth of many pathogens. *L. kunkeei* Fhon2 and *L. apinorum* Fhon13 were not among the producers of hydrogen peroxide, but they did contribute to the production of the volatiles toluene, xylene,

and ethylbenzene. These volatiles are toxic substances to bacteria. Specifically, strain Fhon2 produced the most xylene whereas Fhon13 was the main producer of ethylbenzene. Neither contributed to octane production, and only strain Fhon13 produced a small amount of nonane. Notable, again, was the ability of Fhon13 to produce the highest amount of 2-heptanone among the 13 strains. 2-Heptanone is believed to be an anaesthetic or pheromone which would help alleviate the pain of the patient (Papachristoforou *et al.,* 2012; Olofsson *et al.,* 2016a).

Finally, biofilm formation provides housing of the gut microbiota in honeybees. This was successful even when un-induced *in vitro* by all 13 tested LAB strains. This shows that biofilm production can be used for antimicrobial purposes outside of the honey crop. However, the strains Fhon2 and Fhon13 have a relatively weak ability to form biofilms (Olofsson *et al.,* 2016a; Silva *et al*., 2017). That said, recent studies have identified the *L. kunkeei* strain MP2 as a possibly good candidate due to its biofilm-formation and anti-pathogenic properties (Asenjo *et al.,* 2016; Berríos *et al.,* 2017).

A notable point among the results is that each strain of the LAB symbiont microbiota present in the guts of honeybees contributes its own profile and amount of metabolites. Results confirm that *L. kunkeei* Fhon2 exhibits a highly potent antimicrobial effect and *L. mellifer* Bin4 has the ability to at least inhibit all encountered pathogens. Moreover, the most common free fatty acids are 3-OH C 10:0 and 3-OH C 12:0 which were found in *L. kunkeei* Fhon 2 and Fhon 13, but the proteins did not originate from FLAB lactobacilli (Olofsson *et al.,* 2016a). This is why it is crucial to accurately choose the composition of bacterial species and strains. However, this is compensated by the fact that LAB symbionts have the ability to act against a broad range of pathogens and at low water activity (Olofsson *et al.,* 2016). Thus, as this set has proven effective, it has been replicated and applied against different pathogens both *in vitro* and *in vivo. In vitro* studies were done against mastitis pathogens as well as against pathogens from human chronic infections that are severe and multidrug-resistant. In *in vivo* pilot trials, the mixture including two FLAB among 11 other honeybee gut LAB was tested against hard-to-heal wounds in

horses and Chronic or recurrent rhinosinusitis (CRS) human patients. The results have been quite successful, in general, showing similar or improved effectiveness in comparison to antibiotics (Olofsson *et al.,* 2016). Details regarding the pathogens and treatment strains involved in each experiment can be found in Appendix II.

In all mentioned cases, the results found that the LAB symbiont mixture was quite often more effective than antibiotic treatment, even those that showed antibiotic resistance (Piccart *et al.,* 2016; Olofsson *et al*., 2016b). The study on horse hard-to-heal horse wounds was especially impressive in terms of speed of onset of wound healing and short duration until either considerably significant or full healing occurs. This study strongly suggests that the spray form of honeybee gut LAB mix can be quite successful as a topical treatments. In the case of CRS patients, *in vitro* studies showed promise which was, unfortunately, not validated in the *in vivo* trial (Butler *et al.,* 2016; Mårtensson *et al.,* 2017). The *in vitro* trial by Butler *et al*., mixed the 13 honeybee gut symbionts with heather honey and tested it against CRS pathogens among other chronic wound pathogens. The results were generally positive as the treatment exhibited similar inhibition zones as some antibiotics (Butler *et al.,* 2016). The next step was to apply it a pilot study as was done by Mårtensson et al. (2017). A nasal spray was developed from the same 13 honeybee gut symbionts described by Olofsson et al. in 2016 and administered to patients with CRS without nasal polyps (CRSsNP). Unfortunately, while the spray was tolerated by the patients, it did not influence the symptoms or microbiota in any way (Mårtensson *et al.,* 2017). Therefore, it is important to keep in mind that even promising *in vitro* results do not always translate to practical success. That said, there is overwhelming empirical evidence in support of the use of LAB and FLAB symbionts in topical creams or sprays. Therefore, future research should focus on identifying the causes of failure and tackling the opportunities for success.

Perspectives of industrial production

All FLAB are found in at least one source which is consumed by humans. *F. ficulneus, F. pseudoficulneus*, and *F. tropaeoli* are all involved in the spontaneous fermentation of cocoa beans, while *F. durionis* is involved in that of tempoyak

(Amiza *et al*., 2006; Lefeber *et al*., 2011; Papalexandratou *et al.*, 2011). *L. kunkeei, L. florum, F. durionis, F. ficulneus, F. fructosus, F. pseudoficulneus, F. tropaeoli* play a role in various wines and musts (Mtshali *et al.,* 2012). *L. kunkeei, L. apinorum,* and *F. fructosus* are found in honey which has a reputation for anti-microbial activity (Endo, 2012; Olofsson *et al*., 2016b). Moreover, the aforementioned trio is also found in honeybees and bee products that are widely accepted as having probiotic and immunity boosting properties (Silva *et al.,* 2017; Maeno *et al.,* 2017). This indicates that they can be used as starter cultures to improve the organoleptic properties of certain food products and beverages, and they can be used as functional cultures to others. More specifically, the likely applications of FLAB in food may be in improving the acceptability of fermented Tempoyak, cocoa beans, and wine and palm sap beverages.

Some FLAB can be used alongside other genera and species as starter cultures to improve organoleptic characteristics, allow the mass production of existing products, and standardize product quality. These can also be beneficial in increasing the diversity of existing product ranges by being included in similar products and developing more desirable attributes (Giraffa, 2014). Moreover, a number of FLAB have been linked with antimicrobial and probiotic function which can be investigated in food production and be used as natural preservatives (El-Ghaish *et al.* 2011). Research and development is needed because FLAB have a limited ability to metabolize carbohydrates and a distinct metabolism of phenolic acids which may alter the aroma of the final product as well as provide some probiotic properties (Filannino *et al.,* 2016).

One such example is the industrialization and improvement of the south-east Asian Sambal Tempoyak appetizer which is being produced in a small-scale, traditional way. In 2006 and 2009, experiments were performed to investigate whether the production of Tempoyak under more controlled circumstances yielded an improved product (Amiza *et al*., 2006; Yuliana and Garcia, 2009). Amiza et al. (2006) aimed to find the optimal salt concentration and compare tempoyak produced naturally to one that was pasteurized and inoculated. A 2% salt concentration was

found to best influence the sourness, sweetness, color, and aroma. Also, results showed that the inoculated Tempoyak exhibited more acceptability (Amiza *et al*., 2006). In contrast, Yuliana and Garcia specifically chose *Pediococcus acidilactici* as starter culture and found that the final product had better acceptability than naturally fermented ones (2009). This evidence supports the creation of a set culture which can improve the quality and even quantity of Tempoyak production. Tempoyak industrialization may improve durian fruit farmers' income and facilitate its introduction to other countries. A current obstacle to a large scale production is the short shelf-life of only several months. Finding preservation methods, selling the product in jars instead of aluminum containers, and inoculating the fruit with set cultures are ways to improve the general acceptability and marketability (Yuliana and Garcia, 2009; Owens, 2014).

Another example of product improvement is the attempted integration of *F. fructosus* into wheat bran bread (Prückler *et al.,* 2015). The desired outcome was to reduce the resulting bitter flavor and bran-specific aftertaste. The results did provide some insight into carbohydrate metabolism, but none of the bacteria improved the sensory characteristics. The most notable feature of *F. fructosus* in that experiment was that it was able to grow on wheat bran and produced high acid concentrations of acid and mannitol (Prückler *et al.,* 2015). Different ingredients and combinations should be investigated in light of the lessons learned from this trial.

In the cases of fermented beverages Taberna, Bandji, Tempranillo and other wines, these FLAB play a secondary role in flavor development and fermentation facilitation (Alcantara-Hernandez *et al*., 2010; Papalexandratou *et al*., 2011; Ouoba *et al*., 2012; González-Arenzana *et al.,* 2015). This collective of FLAB produces large amounts of mannitol from glucose and fructose fermentation which leads to the production of a sweet flavor and acid which reduces the growth of spoilage bacteria as well as imparting a sour flavor to the resulting product. Again, the standardization of starter cultures could help produce a consistent product with better acceptability.

Appendix

Chemical products

Recent studies have shown that FLAB can be useful in the production of increasingly demanded chemicals - namely lactic acid, mannitol, and erythritol. Even though it seems to be less vital than the other possibilities of FLAB use, there are highly varied uses for each chemical. Lactic acid is a multipurpose chemical in food, cosmetic, chemical, and pharmaceutical industries. Mannitol is a sugar alcohol that is used as an osmotic, diuretic medicine and as a natural sweetener. Erythritol is used as a natural sweetener in foods and beverages (Vijayakumar *et al.,* 2008; Tyler *et al.,* 2016; Rodriguez *et al.,* 2017).

The importance of focusing on lactic acid production lies in the fact that its world market is increasing every year. It is classified as GRAS and is widely used in food and pharmaceutical industries. It was previously prepared by using refined simple sugars and starch materials. However, this is an expensive method of production even though it results in a purer form of lactic acid. In order to reduce the cost and resulting pollution, efforts are being focused on the upcycling of wastes from kitchens, food processing plants, leftover carbohydrate-based foods such as crops, wheat stalk, and bran, and wastewater sludge (Vijayakumar *et al.,* 2008). For FLAB, fructose and glucose in the right aerobiosis are required (Endo, 2012). Moreover, the use of FLAB will surely yield predominantly singular forms of lactic acid as opposed to the chemical production which always yields a mixture of the isomers and would require further processing. In food industry, lactic acid is important in confectionary, beer and beverage production, olives and pickles, dairy production, and meat products. In each of these, it is used as an acidulant, a preservative, flavor influencer, microbiota regulator, fermentation time reducer, and appearance enhancer. In addition, it is a natural ingredient in cosmetics, and it is highly important for chemical conversions. It also acts as a descaling agent, solvent, cleaning agent, antimicrobial agent, and humectant. In pharmaceutical industry, it is used as an electrolyte in intravenous solutions, in artificial kidney machines, prostheses, and surgical sutures thanks to its biodegradability. It is also relevant to the production of topical ointments and creams. Next up is its use in biodegradable

commodity plastics, cellophane production for food packaging, textile printing, and many other uses (Vijayakumar *et al.,* 2008).

In order to assess feasibility, practical research must be done. Lactic acid production yield and efficiency depends on factors relevant to the substrate, conditions, and fermenting bacteria. The purity of the sugar substrate(s), presence of nitrogen sources, mineral salts, and carbon sugars all improve the yield and speed of production. Moreover, the temperature at which fermentation occurs is species-dependent, the optimal pH is generally between five and seven, and 5.7 in the case of lactobacilli. All FLAB are known to grow well within the specified range, except for *F. durionis* which needs further investigation. There are too many unknowns to determine exactly how each FLAB would react (Vijayakumar *et al.,* 2008).

Mannitol and erythritol are also growing in market size because they are noncariogenic and nonglycaemic. There is also some evidence pointing to their role as food or functional substrates for plant bacteria (Tyler *et al.,* 2016; Rodriguez *et al.,* 2017). As in the case of lactic acid, the carbon sugar substrates affect the yield and efficiency of fermentation. *L. florum* 2F *and F. tropaeoli* CRL 2034 have especially gained attention for their advanced ability to produce polyols like mannitol and erythritol (Filannino *et al.,* 2018). Unfortunately, such applications do not seem likely to be widespread in industry. In general, *E. coli* and *Bacillus* species are the preferred bacteria for industrial production. This is because using LAB is more costly due to their higher nutrient requirement compared to *E. coli* and *Bacillus*. Such endeavors require further cost analyses.

Mannitol is being used in pharmaceutical industry as an osmotic diuretic and in food industry targeting diabetics. Its low mold ability and good wetting characteristics alongside its good water solubility and adequate insensitivity to humidity make it an attractive option for manufacturers. This has led to a large world market size that is expected to double during the next decade. (Rodriguez *et al.,* 2017). What has been hindering growth is that the current production processes yield a mixture of only 25:75 mannitol:sorbitol which is expensive to separate.

Therefore, attention is being turned to reduce its cost of production while increasing mannitol's yield and purity. On one hand, using *F. tropaeoli*, *F. fructosus, F. pseudoficulneus,* and other LAB is beneficial because it is an option which immediately introduces mannitol into the food product. On the other hand, it is still possible to isolate and purify it easily for pharmaceutical and food industry (Rodriguez *et al.* 2017).

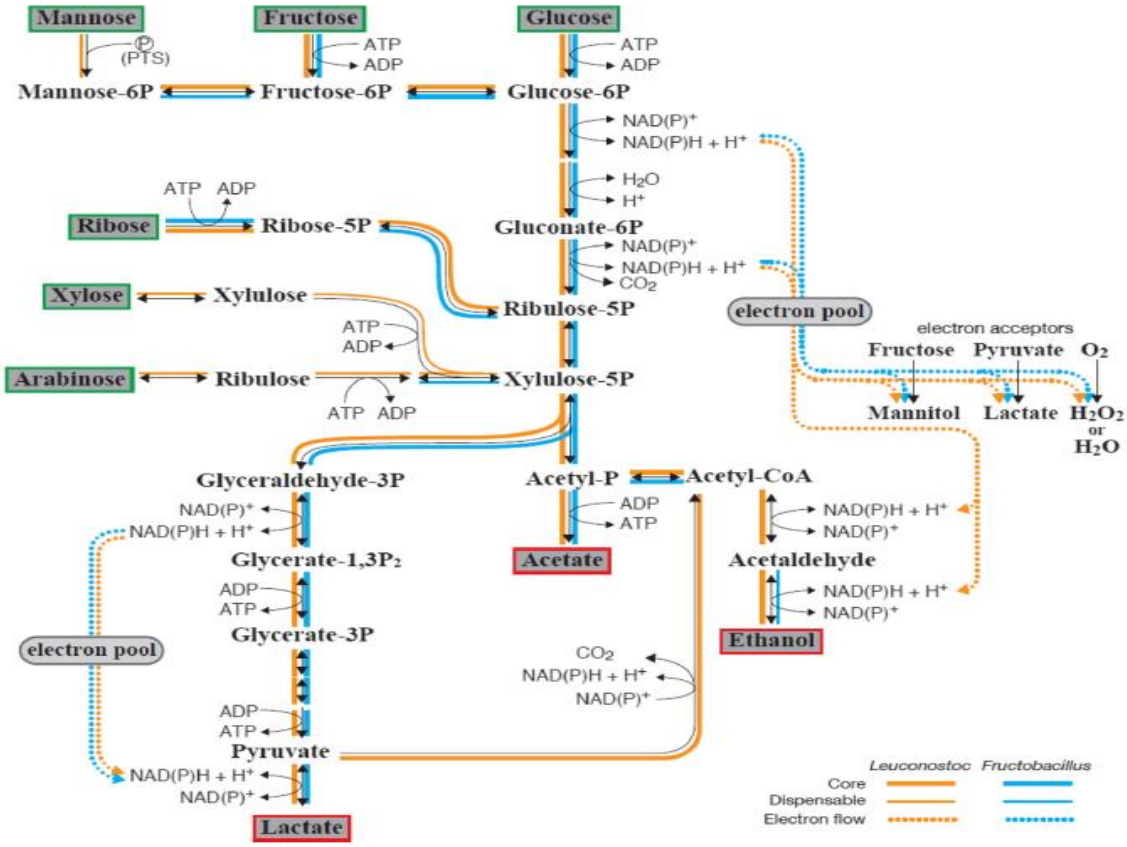
F. tropaeoli CRL 2034 isolated from figs produced record-breaking amounts of mannitol and established itself as the best producer amongst the strains tested by Rodriguez et al. (2017). It could grow under high osmotic pressure, it can be used for scaling up the production process. The analysis of the process was performed using Response Surface Methodology with Central Composite Design based on Total Saccharide Content, testing osmotic stress, and stirring, testing oxidative stress, to detect any further optimization margins. The *F. tropaeoli* CRL 2034 isolate synthesized approximately 100 g/l after 24 hr of incubation. Results found that TSC is a significant, positive factor whereas stirring is not (Rodriguez *et al.* 2017). Unfortunately, such production from bacteria is expensive and does not produce as high quantities as current industrial methods given that polyols are not major products.

Erythritol is gaining attention because it can be used instead of other sweeteners because while being nearly non-caloric, consuming it does not pose the risk of laxative effect. Like lactic acid and mannitol, the current production processes are expensive and there is a need to drive the cost down. Cheap erythritol originating from a naturally occurring, food grade microbe such as *L. florum* 2F would be preferred over the current production from yeast fermentation (Tyler *et al.,* 2016). Unfortunately, the production of erythritol is not sufficient enough to make FLAB a relevant source for industrial production.

Metabolic functions related to NAD(P)+ regeneration and ATP production lead to the synthesis of the different polyols. The composition of the starting sugar substrate determines the metabolism rate and effectiveness. When *L. florum* 2F was cultured on a suitable medium such as mMRS and a glucose and fructose mixture,

the main by-products were a mixture of erythritol and mannitol. That said, the most efficient growth and rate are observed when fructose is the only available carbon source (Tyler *et al.,* 2016). Moreover, mannitol is the predominant product even though the amount is not as high as in the case of *F. tropaeoli*. The next steps could be further genome characterization and a process optimization study similar to the one performed by Rodriguez et al. (2017) for *F. tropaeoli.* It would be useful to find genes relevant to erythritol biosynthesis to understand the metabolic drivers. These can be useful when investigating the fermentation parameters. The pH, temperature, mineral salts, starting sugar composition, and levels of exogenous pantothenate are some of the main factors that can be adjusted (Tyler *et al.,* 2016).

Fig. 3: Predicted sugar metabolic pathways showing core and dispensable genes (in colors) as well as electorn flow (in dotted lines) in *Fructobacillus* spp. and *Leuconostoc* spp. (Endo *et al.,* 2015)



Appendix

From Regulation (EC) 258/97 on novel foods (NF) and novel food ingredients

From Article 3, Definitions:

1. For the purposes of this Regulation, the definitions laid down in Articles 2 and 3 of Regulation (EC) No 178/2002 apply.
2. The following definitions also apply:
   a. **'novel food' means any food that was not used for human consumption to a significant degree within the Union before 15 May 1997, irrespective of the dates of accession of Member States to the Union, and that falls under at least one of the following categories:**
      i. food with a new or intentionally modified molecular structure, where that structure was not used as, or in, a food within the Union before 15 May 1997;
      ii. **food consisting of, isolated from or produced from microorganisms, fungi or algae;**
      iii. food consisting of, isolated from or produced from material of mineral origin;
      iv. food consisting of, isolated from or produced from plants or their parts, except when the food has a history of safe food use within the Union and is consisting of, isolated from or produced from a plant or a variety of the same species obtained by:
         - traditional propagating practices which have been used for food production within the Union before 15 May 1997; or
         - non-traditional propagating practices which have not been used for food production within the Union before 15 May 1997, where those practices do not give rise to significant changes in the composition or structure of the food affecting its nutritional value, metabolism or level of undesirable substances;
      v. food consisting of, isolated from or produced from animals or their parts, except for animals obtained by traditional breeding practices which have been used for food production within the Union before 15 May 1997 and the food from those animals has a history of safe food use within the Union;
      vi. food consisting of, isolated from or produced from cell culture or tissue culture derived from animals, plants, micro-organisms, fungi or algae;
      vii. food resulting from a production process not used for food production within the Union before 15 May 1997, which gives rise to significant changes in the composition or structure of a food, affecting its nutritional value, metabolism or level of undesirable substances;
   b. **'history of safe food use in a third country' means that the safety of the food in question has been confirmed with compositional data and from experience of continued use for at least 25 years in the customary diet of a significant number of people in at least one third country, prior to a notification referred to in Article 14;**

14. Appendix II

The appendix II contains quick details related to the experiments noted in the text including the bacterial strains used and encountered pathogenic bacteria.

Carbohydrate Fermentation by FLAB:

Tab. 6: Details related to the fermentation of different carbohydrates by FLAB (Endo and Dicks, 2014)

	Fru. tropaeoli	*Fru. durionis*	*Fru. ficulneus*	*Fru. fructosus*	*Fru. pseudoficulneus*	*Lb. kunkeei*	*Lb. florum*
Acids from:							
D-Fructose	1 day[a]	1 day	1 day	1 day	1 day	1 day	1 day
D-Glucose	2 days	3 days	4 days	4 days	3 days	3 days	2 days
D-Mannitol	5 days	5 days	6 days	6 days	6 days	5–6 days or w	–
Sucrose	–	3 days	w	–	–	2–3 days	–
D-Turanose	–	4 days	w	–	–	v	–
D-Ribose	–	w	–	–	–	–	–
Gluconate	–	w	w	–	–	w or –	–
Methyl-α-D-glucopyranoside	–	6 days	w	–	–	–	–
Maltose	–	7 days	w	–	–	–	–
Trehalose	–	6 days	5 days	–	–	–	–
Enzyme activities (determined by APIZYM):							
Alkaline phosphatase	+	w	+	w	w	ND	ND
Butyrate esterase (C4)	–	–	w	–	w	ND	ND
Myristate lipase (C14)	–	–	–	–	w	ND	ND
Trypsin	–	w	w	–	w	ND	ND
Chymotrypsin	+	+	+	w	w	ND	ND
α-Galactosidase	–	w	w	–	w	ND	ND

API 50 CHL was used for carbohydrate fermentation. Carbohydrates not listed here are negative by all species.
All these strains showed positive reaction to enzyme activities from acid phosphatase and phosphoamidase and negative reactions to enzyme activities from caprylate esterase (C8), leucine, valine and cysteine aminopeptidases, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, β-glucosaminidase, α-mannosidase and α-fucosidase.
+, positive; –, negative; v, variable; w, weakly positive; ND, no data available; *Lb.*, *Lactobacillus*.
[a]Days needed for fermentation.

Appendix

Quick details about the experiment on human severe, drug-resistant pathogens (Olofsson *et al*., 2016b):

Tab. 7: Tested pathogens and treatment strains in the experiment of Olofsson *et al.*, 2016a

Tested pathogens	Treatment LAB strains
Acinetobacter A23 Z32524	*Lactobacillus helsingborgensis* Bma5
Candida albicans	*L. kimbladii* Hma2
Citrobacter freundii CR01 5A	*L. mellis* Hon2
Enterobacter cloacae JSB 5B	*L. mellifer* Bin4
Enterococcus faecalis E12 VRE	*L. melliventris* Hma8
Escherichia coli V517	*L. apis* Hma11
Klebsiella aerogenes Clmp R	*L. kullabergensis* Biut2
Klebsiella oxytoca JSB 5B	*L. apinorum* Fhon13
MRSA clinical isolate 18	*L. kunkeei* Fhon2
Pseudomonas aeruginosa LE08	*Bifidobacterium* sp. Bin7
Serratia narcescens NJ19 5c	*Bifidobacterium* sp. Hma3
Staphylococcus areus FJ02	*Bifidobacterium asteroides* Bin2
Staphylococcus areus 74022 PR	*Bifidobacterium coryneforme* Bma6
Staphylococcus areus CR01	

LAB strains from the honey stomach of

- the dwarf honeybee *Apis andreniformis*,
- the giant honeybee *Apis laboriosa*
- the stingless bee *Melipona beechii*

Tab. 8: Bioactive substances produced by LAB from *Apis mellifera* honeybees (mg/sample) (Olofsson *et al.*, 2016a)

Genus	Strain	Acetic acid	Formic acid	Lactic	H_2O_2	Benzene	Toluene	Octane	Ethylbenzene	Xylene	Nonane
Lactobacillus	Fhon2	>263	>17	680		0·0045	0·004	0·0	0·0022	0·39	0·0
Lactobacillus	Fhon13	>327	>28	600		0·0018	0·008	0·0	0·031	0·29	0·0068
Lactobacillus	Hma11	>306	>16	500	+	0·0005	0·036	0·027	0·0	0·23	0·0127
Lactobacillus	Hon2	>290	>16	770		0·001	0·045	0·049	0·0004	0·28	0·02
Lactobacillus	Bin4	161·8	9·3	600		0·074	0·0	0·0	0·017	0·01	0·0
Lactobacillus	Hma2	>271	>16	710	+	0·0003	0·057	0·049	0·0	0·25	0·0127
Lactobacillus	Bma5	>267	>16	900	+	0·0004	0·046	0·059	0·004	0·28	0·0163
Lactobacillus	Hma8	206·4	12·7	1060	+	0·0008	0·07	0·049	0·0005	0·24	0·02
Lactobacillus	Biut2	>258	>14	950	+	0·0006	0·036	0·039	0·0004	0·26	0·0159
Bifidobacterium	Bin2	>302	>20	260		0·0002	0·040	0·369	0·003	0·27	0·0147
Bifidobacterium	Bin7	>297	>25	420		0·009	0·045	0·579	0·004	0·25	0·02
Bifidobacterium	Hma3	>294	>20	220		0·0014	0·040	0·559	0·004	0·26	0·02
Bifidobacterium	Bma6	208·2	13·0	260		0·0005	0·0	0·419	0·003	0·01	0·0
Summation	All 13 LAB	>3451	>223	7930		0·094	0·427	2·198	0·0695	3·011	0·1594

Appendix

Details of the experiment on the wound healing of horses (Olofsson *et al.*, 2016b):

Tab. 9: Encountered pathogens and specific LAB combination tested by Olofsson *et al.*, 2016b on horses

Tested pathogens	Treatment LAB strains
Staphylococcus (12 species) *Corynebacterium* (5 species) *Streptococcus* (5 species) Acinetobacter genera	*Lactobacillus kunkeei* Fhon2 *L. apinorum* Fhon13 *L. mellifer* Bin4 *L. mellis* Hon2 *L. kimbladii* Hma2 *L. melliventris* Hma8 *L. helsingborgensis* Bma5 *L. kullabergensis* Biut2 *L. apis* Hma11 *Bifidobacterium* sp. Bin7 *Bifidobacterium* sp. Hma3 *Bifidobacterium asteroides* Bin2 *Bifidobacterium coryneforme* Bma6

- The most rapid cases, the wounds began to heal after the first application.
- The wounds were treated every 2 days.
- The mean healing time was 16 days.
- Bacterial identification by 16S rRNA gene sequencing.
- Painless healing of hard-to-heal equine wounds were treated or cured

Tab. 10: Bioactive metabolites produced by *L. kunkeei* and *L. apinorum* in fresh honey (Olofsson et al., 2016b)

Metabolite	Action	Role in wound healing
Organic acids: Formic acid, lactic acid, acetic acid	Lower pH	Acidification kills pathogens
Toxic volatiles: Benzene, Hydrogen peroxide (H_2O_2), nonane	Solvent, obstruction of the bacterial membranes	Increase rate of wound closure and rate of epithelisation
2-Heptanone	local anaesthetic	Honeybee pheromone, reduces pain

Appendix

Chronic or recurrent rhinosinusitis (CRS): test on humans in nasal spray form (Mårtensson *et al.*, 2017):

Tab. 11: Pathogens and treatment strains encountered during the CRS experiment by Martensson *et al.*, 2017

Tested pathogens	Treatment LAB strains
Actinomyces odontolyticus	*Lactobacillus kunkeei* Fhon2N
Bacillus sonorensis	*L. apinorum* Fhon13
Brevibacterium casei	*L. mellifer* Bin4N
Citrobacter freundii	*L. mellis* Hon2N
Clavispora lusitaniae	*L. kimbladii* Hma2N
Corynebacterium	*L. melliventris* Hma8N
propinquum/pseudodiphtheriticum/simulans/species	*L. helsingborgensis* Bma5N
Enterobacter aerogenesis	*L. kullabergensis* Biut2N
Enterococcus faecalis Escherichia coli	*L. apis* Hma11N
Gemella haemolysans	*Bifidobacterium* sp. Bin7N
Haemophilus haemolyticus	*Bifidobacterium* sp. Hma3N
Haemophilus influenzae	*Bifidobacterium asteroides* Bin2N *Bifidobacterium*
Klebsiella oxytoca	*coryneforme* Bma6N
Klebsiella pneumoniae	
Kocuria kristinae	
Lactobacillus kunkeei	
Micrococcus luteus	
Moraxella_sg_Branhamella catarrhalis	
Moraxella_sg_Moraxella lincolnii	
Moraxella_sg_Moraxella nonliquefaciens	
Neisseria perflava	
Neisseria subflava	
Propionibacterium acnes	
Propionibacterium avidum	
Propionibacterium species	
Pseudomonas aeruginosa	
Rothia amarae	
Rothia mucilaginosa	
Serratia marcescens	
Serratia ureilytica	
Staphylococcus aureus	
Staphylococcus capitis	
Staphylococcus epidermidis	
Staphylococcus haemolyticus	
Staphylococcus hominis	
Staphylococcus intermedius	
Staphylococcus lugdunensis	
Staphylococcus pasteuri	
Staphylococcus succinus	
Staphylococcus warneri	
Streptococcus gordonii,	
Streptococcus infantis	
Streptococcus oralis/mitis/peroris/parasanguinis/	
pneumoniae/pseudopneumoniae group	
Streptococcus salivarius	
Streptococcus sanguinis	
Streptococcus urinalis	
Streptococcus vestibularis	

- In this study including patients with CRS without nasal polyps (CRSsNP)

Appendix

The composition of honey as listed by Silva *et al*., 2017:

Tab. 12: Listing of the chemical components of honey as found in Silva *et al.,* 2017

Phenolic acids:	**Antimicrobial activity:**
caffeic	carbon
ellagic	lipids
ferulic	amino acids
p-coumaric acids	proteins
	vitamins
Flavonoids:	minerals
apigenin	**Healing effect:**
chrysin	
galangin	hydrogen peroxide
hesperetin	high osmolarity
kaempferol	acidity
pinocembrin	non-peroxide factors
quercetin	nitric oxide
	phenols
Antioxidants:	
tocopherols	
ascorbic acid	
superoxide dismutase	
catalase	
reduced glutathione	

- These compounds are known for their ability to reduce free radicals
- Composition may vary depending on the microbiota source

15. List of abbreviations

CCD: Colony Collapse Disorder

EFB: European foulbrood

EFFCA: European Food and Feed Cultures Association

GRAS: Generally Regarded as Safe

IDF: International Dairy Federation

QPS list: Qualified Presumption of Safety

Glossary

Banji: A palm wine made from fermented palm sap, as named in Burkina Faso. It is a traditional beverage in different parts of Africa under different names.

Beebread: The food consumed by adult bees and larvae. A mixture of pollen and nectar which bees pack into hexagonal wax combs in the hive.

Crop, foregut, or honey stomach: A bag that allows the bee to store nectar and transport it.

Colony Collapse Disorder: A phenomenon that occurs when the majority of worker bees in a colony disappear and leave behind a queen. The bees do not die in the colony.

European foulbrood: A fatal honeybee disease caused by the bacterium *Melissococcus plutonius.*

GRAS: Generally Regarded as Safe: A classification of food-related ingredients in the USA based on a continued history of safe use.

Musts: Young wines, freshly pressed grape juice.

Taberna: An alcoholic whitish drink traditionally produced in Mexico from the natural fermentation of the sap of the *Acrocomia aculeate* coyol palm tree.

Tempoyak: A condiment traditionally consumed in Malaysia and Indonesia made from fermented durian fruit.

Tempranillo: A variety of black grapes originating from Spain, used to make wine.