Biofilm formation as an extra gear for *Apilactobacillus kunkeei* to counter the threat of agrochemicals in honeybee crop

Ali Zein Alabiden Tlais,1,† Andrea Polo1,*,† Pasquale Filannino,2 Vincenzo Cantatore,2 Marco Gobbetti1 and Raffaella Di Cagno1

1Faculty of Sciences and Technology, Libera Università di Bolzano, Bolzano, Italy.
2Department of Soil, Plant and Food Science, University of Bari Aldo Moro, Bari, Italy.

Summary

The alteration of an eubiosis status in honeybees’ gut microbiota is directly linked to the occurrence of diseases, and likely to the honeybees decline. Since fructophilic lactobacilli were suggested as symbionts for honeybees, we mechanistically investigated their behaviour under the exposure to agrochemicals (Roundup, Mediator and Reldan containing glyphosate, imidacloprid and chlorpyrifos-methyl as active ingredients respectively) and plant secondary metabolites (nicotine and *p*-coumaric acid) ingested by honeybees as part of their diet. The effects of exposure to agrochemicals and plant secondary metabolites were assessed both on planktonic cells and sessile communities of three biofilm-forming strains of *Apilactobacillus kunkeei*. We identified the high sensitivity of *A. kunkeei* planktonic cells to Roundup and Reldan, while cells embedded in mature biofilms had increased resistance to the same agrochemicals. However, agrochemicals still exerted a substantial inhibitory/control effect if the exposure was during the preliminary steps of biofilm formation. The level of susceptibility resulted to be strain-specific. Exopolysaccharides resulted in the main component of extracellular polymeric matrix (ECM) in biofilm, but the exposure to Roundup caused a change in ECM production and composition. Nicotine and *p*-coumaric acid had a growth-promoting effect in sessile communities, although no effect was found on planktonic growth.

Introduction

Among pollinators, social honeybees are of great interest as they contribute both directly to human diets (e.g. through propolis, royal jelly, honey and pollen), and indirectly by providing pollination services to a wide range of crops and wild plants of human impact (Hristov et al., 2020a). It was estimated that 84% of the European Union’s crops, 90% of wild plants and 75% of feed productions depend, at least in part, on honeybees as most important crop pollinators (Potts et al., 2010) and, overall, 35% of the global food production comes from crops relying on them (Hamdi et al., 2011). Besides this economic value, the contribution of bee pollination has to be considered also within the overall biodiversity that has an inestimable economic value (Hristov et al., 2020a). The population of domesticated honeybees is generally increasing (Aizen and Harder, 2009). Nevertheless, in recent years, a severe decline in bee populations and related economic damage has been reported (Potts et al., 2010; Hristov et al., 2020b). Social insects, such as ants and bees, appear to be particularly sensitive to changes in their social environment (Kohlmeier et al., 2016). The prevention of honeybee colony losses association (COLOSS) noticed a loss of production colonies of 20.9% in winter of 2016/2017 in many European and some non-European countries (Brodschneider et al., 2018). The drivers of losses are numerous pressures mainly due to anthropogenic reasons such as the decline of flowers’ abundance and diversity, the chronic exposure to cocktails of agrochemicals, and, simultaneously, to novel pests and parasites (Hristov et al., 2020b). The phenomenon known as colony collapse disorder (CCD), a syndrome characterized by the rapid disappearance from a colony of its adult bee population, also contributed to the decline. The scenario seriously concerns the future of nutritious food productions, and it represents a significant threat to the integrity of biodiversity, to global food webs and to human health (Cox-Foster et al., 2007).

Relatively recent studies highlighted that the alteration of an eubiosis status in honeybees gut microbiota is...
directly linked to the occurrence of diseases. As for humans, a protective gut microbiome inhabiting honeybees contributes, under unstressed conditions, to erect a barrier against diseases, while an unbalanced microbiome (i.e. intestinal dysbiosis) is associated to a compromised health status (e.g. pathogen invasions) (Anderson and Ricigliano, 2017). Among stressed conditions, the oral exposure to specific agrochemicals (e.g. glyphosate, thiamethoxam [neonicotinoid], imidacloprid [neonicotinoid], fipronil [phenylpyrazole], boscalid [carboxamide]; chlorpyrifos-methyl [phosphorganic]) poses a serious threat to the structure and composition of honeybee intestinal community, in a dose-dependent manner (Favaro et al., 2019; Rouzé et al., 2019; Motta and Moran, 2020; Paris et al., 2020). The capacity of agrochemicals to affect the microbial communities naturally co-evolving with honeybees may be much higher than currently appreciated (Daisley et al., 2020a; Cuesta-Maté et al., 2021). As a consequence, promising strategies aiming to support honeybees’ health status were proposed based on the management of the natural eubiotic bee microbiome (Baffoni et al., 2016; Daisley et al., 2020a). Fructophilic lactobacilli like *Fructobacillus fructosus*, *Apilactobacillus apinorum* and, especially, *Apilactobacillus kunkeei* are inhabitants of honeybee crop, which is located between oesophagus and proventriculus (Anderson et al., 2011, 2013; Corby-Harris et al., 2014; Filannino et al., 2016). As they account for a relatively small proportion of the overall gut microbiota, the functional role of fructophilic lactobacilli in the crop was neglected and poorly investigated compared to others’ core phylotypes (Gillumella, Snodgrassella, Lactobacillus Firm-4 and Firm-5 and Bifidobacterium). Nevertheless, a role for fructophilic lactobacilli in improving barrier function, reducing pathogen load and facilitating various metabolic processes was proposed by some researchers, likely resulting into a symbiotic relationship in honeybee gut (Forsgren et al., 2010; Filannino et al., 2016; Arredondo et al., 2018; Lamei et al., 2019; Daisley et al., 2020b; Iorizzo et al., 2020; Bielik et al., 2021). Fructophilic lactobacilli were also suggested as resources for paratransgenesis, which uses genetically modified symbiotic microbes to improve host health and pathogen resistance (Rangberg et al., 2012, 2015; Maddaloni et al., 2014). As reported by Rangberg et al. (2015), transformed *A. kunkeei* cells survived well in the honeybee midgut upon reintroduction.

Crop is the honeybee’s nutritional interface with the environment and colony food stores. The main feed of honeybees is pollen, which is easily contaminated by agrochemicals or water that indirectly contains agrochemicals. On the other side, plant secondary metabolites within pollen hold antimicrobial features and may therefore have a role in modulating the honeybee gut microbiota (Manson et al., 2010; Engel et al., 2012). Thus, fructophilic lactobacilli inhabiting the honeybee crop are strongly affected by diet and diet-driven changes. According to Cuesta-Maté et al. (2021), the most far-reaching implications of pesticide exposure fall mainly on crop bacteria, like *A. kunkeei*. This study aimed to investigate the effects of pollen/plant secondary metabolites (ingested by bees as part of their diet) and agrochemicals (associated with honeybee death) on both planktonic cells and microbial biofilm of fructophilic lactobacilli. For biofilm, a focus on both the preliminary phases of biofilm growth and on already developed biofilms was provided. Lactobacilli are able to colonize the honeybee crop and form biofilms and networks, as proved in vivo by Vásquez et al. (2012), but the attachment and biofilm formation as well as the resulting protective effects is reliant on still unknown mechanisms. Understanding of bacterial reaction to stressful diet components might clarify the real beneficial potential of fructophilic lactobacilli for honeybees’ health and help to identify drivers of honeybees decline and effective countermeasures. Furthermore, our study will expand the knowledge on specific niche-adaptation mechanisms adopted by fructophilic lactobacilli, which still represent a partially unexplored bacterial taxa (Filannino et al., 2016).

**Results**

**Kinetic of growth and carbohydrates consumption**

Fructophilic lactobacilli strains were initially screened based on the kinetics of growth and carbohydrates reduction in synthetic media supplemented with fructose or sucrose as major carbon source. After 24 h of incubation in the medium containing fructose, the turbidity (A) increased up to 0.43–0.71 Ab620 units. Maximum growth rate (µmax) and lag phase (λ) ranged from 0.07 ± 0.00 to 0.13 ± 0.01 Ab620 units h⁻¹ and from 4.48 ± 0.12 to 9.66 ± 0.04 h respectively. Although all analysed strains demonstrated almost complete reduction of fructose, *A. kunkeei* BV61 reached the highest values of turbidity (0.71 ± 0.01 Ab620 units). In the medium containing sucrose, only *A. kunkeei* strains were able to grow and utilize sucrose. The greatest reduction of sucrose was reached by *A. kunkeei* BV61 (32.6 ± 4.79%). After sucrose reduction, fructose was not detected using any of *A. kunkeei* strain, and only traces amount of glucose were detected. Values of A, µmax and λ were in the range of 0.55–0.62 Ab620 units, 0.05–0.06 Ab620 units h⁻¹ and 6.20–7.56 h respectively (Table 1).

**Exopolysaccharide production**

The EPS production by fructophilic lactobacilli on both solid and liquid media was qualitatively (visual inspection) and quantitatively assessed. Visual inspection of cultures distinguished *A. kunkeei* strains as efficient producers of
Table 1. Growth parameters, carbon sources utilization and exopolysaccharides (EPS) production in fructose and sucrose synthetic media inoculated with Fructobacillus fructose PL22, PL10 and Apilactobacillus kunkeei PL13, BEE4 and BV61, which were incubated for 24 h at 30°C.

| Samples          | A  | i  | EPS (g l⁻¹) | Fructose reduction (%) | Sucrose reduction (%) | Fructose production (g l⁻¹) | Sucrose production (g l⁻¹) |
|------------------|----|----|-------------|------------------------|----------------------|-----------------------------|----------------------------|
| Fructobacillus fructose PL10 | 0.43 ± 0.01 ab | 0.07 | 0.00 a | 4.93 ± 0.05 b | 97.75 ± 0.23 b | 9.68 ± 0.04 b |
| F. fructose PL25 | 0.51 b | 0.01 d | 0.08 | 0.08 a | 97.89 ± 0.23 b | 0.12 c | 100.00 a |
| A. kunkeei PL10 | 0.31 a | 0.04 a | 0.08 | 0.01 b | 99.73 ± 0.00 a | 0.16 b | 0.15 a |
| A. kunkeei BEE4 | 0.64 ± 0.00 ab | 0.13 | 0.00 a | 9.66 ± 0.04 b | 100.00 b | 0.15 |
| A. kunke ei BV61 | 0.71 ± 0.01 a | 0.13 | 0.01 a | 8.32 ± 0.31 b | 100.00 a | 0.05 | 0.02 |

Data are the mean of three separate analyses ± standard deviations. Means within the columns with different letters are significantly different (P < 0.05). Bold indicates the highest value. “Ab620” was the absorbance at 620 nm, “Amax” was the maximum value of absorbance, “i” was the length of the lag phase (h), and “t” was the time (h). The symbol “ab” represents not detected.

Effect of plant secondary metabolites and agrochemicals on planktonic cells

None of the A. kunkeei strains was able to grow when exposed to Roundup or Reldan at examined concentrations (Fig. 1). Compared to the control, the growth patterns of A. kunkeei BEE4, BV61 and PL13 were almost the same upon nicotine, p-coumaric or Mediator treatments with slight differences in the lag phases (Fig. S2).

Effect of plant secondary metabolites and agrochemicals on already developed biofilm

By a preliminary visual inspection, no differences of size, morphology and stickiness were observed on biofilm after 96 h of their growth. Through CLSM observations, all biofilms appeared as widespread agglomerates of bacterial cells (green fluorescence) attached to the surface and embedded in ECM containing EPS (red fluorescence) (Fig. 2). Biofilm architecture was strain-dependent: A. kunkeei PL13 produced characteristic mushroom structures (panels M–R), while such structures were less pronounced in A. kunkeei BEE4 and BV61 biofilms (panels A–F and G–L, respectively).

Developed biofilms were further incubated for 96 h on substrate supplemented with plant secondary metabolites and agrochemicals, and the cell density and biomass of biofilms were measured and compared to control samples (Fig. 3A and B). All fructophilic lactobacilli strains had a significant (P < 0.05) reduction of cell density after exposure to both Reldan and Roundup compared to biofilm without exposure (control), with Reldan showing the highest reduction effect. When treated with Reldan, the cell density decreased from 8.25 ± 0.12 to 7.11 ± 0.15, from 8.10 ± 0.16 to 6.00 ± 0.00 and from 7.63 ± 0.05 to 6.00 ± 0.00 Log CFU g⁻¹ for A. kunkeei BEE4, BV61 and A. kunkeei PL13, respectively. The results showed a significant reduction in the cell density of all the investigated strains, which was more pronounced in biofilms exposed to Reldan compared to Roundup.

The EPS production of A. kunkeei PL13, BEE4 and BV61 was significantly reduced after exposure to both Reldan and Roundup compared to the control. The EPS production was highest in A. kunkeei PL13, followed by A. kunkeei BEE4 and BV61, respectively. The results showed that both Reldan and Roundup could significantly reduce the EPS production of A. kunkeei strains, which was more pronounced in A. kunkeei PL13.

Conclusions

The results indicated that A. kunkeei strains had a potential to produce EPS as a protective layer against plant secondary metabolites and agrochemicals. The EPS production was significantly reduced after exposure to both Reldan and Roundup, which could be attributed to the binding of EPS to the plant secondary metabolites and agrochemicals, reducing their availability to the bacteria. The results also showed that the EPS production was more pronounced in A. kunkeei PL13, which could be attributed to the higher EPS production of this strain.

References

1. Cho, Y. H., Kim, S. Y., and Kim, D. H. (2015). Growth parameters, carbon sources utilization and exopolysaccharides (EPS) production in fructose and sucrose synthetic media inoculated with Fructobacillus fructose PL22, PL10 and Apilactobacillus kunkeei PL13, BEE4 and BV61, which were incubated for 24 h at 30°C. Microbial Biotechnology 2022; The Authors.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2019R1A6A1A11035833).

Conflict of interest

The authors declare no conflict of interest.
and PL13 respectively. When treated with Roundup, the cell density was 7.32 ± 0.15, 7.11 ± 0.15 and 7.14 ± 0.25 Log CFU g⁻¹ for A. kunkeei BEE4, BV61 and PL13 respectively. The exposure to Mediator caused significant (P < 0.05) reduction of Log CFU g⁻¹ only for A. kunkeei BEE4 (from 8.25 ± 0.12 to 7.85 ± 0.30 Log CFU g⁻¹). An opposite effect was found with nicotine, which induced a significant (P < 0.05) increase of A. kunkeei BV61 and PL13 cell density. A significant (P < 0.05) increase of A. kunkeei BV61 cell density was also found in presence of p-coumaric acid. Overall, after exposure to nicotine, Log CFU g⁻¹ reached the values of 8.51 ± 0.13, 8.61 ± 0.12 and 8.09 ± 0.07 Log CFU g⁻¹ for A. kunkeei BEE4, BV61 and PL13, respectively, while the cell densities after exposure to p-coumaric acid increased to 8.44 ± 0.12 and 8.56 ± 0.09 Log CFU g⁻¹ for A. kunkeei BEE4 and BV61 respectively (Fig. 3A). Despite the significant changes of the cell densities, the exposure to different agents did not cause significant (P > 0.05) variations of biomass (i.e. biofilm weight) for all strains. The only exception was A. kunkeei PL13 after exposure to Reldan treatment, which had a significant (P < 0.05) decrease of biomass compared to control samples (from 10.25 ± 0.00 to 5.25 ± 0.00 mg). In general, the values ranged from 8.25 ± 0.00 to 12.75 ± 2.3, from 7.25 ± 0.00 to 11.5 ± 1.18 and from 5.25 ± 0.00 to 10.25 ± 0.00 mg for A. kunkeei BEE4, BV61 and PL13 respectively (Fig. 3B).

**Effect of plant secondary metabolites and agrochemicals during biofilm growth**

The influence of plant secondary metabolites and agrochemicals on biofilm growth is shown in Figs 4-6. Visual inspection demonstrated that the exposure to Reldan and Roundup drastically reduced the biofilm growth of all A. kunkeei strains (Fig. 4). On the contrary, apparently the presence of nicotine, p-coumaric acid and Mediator did not affect the macroscopic morphology and size of biofilms compared to controls (Fig. 4). Figure 5 shows the biofilms observed by CLSM. All strains were able to develop biofilm even if exposed to nicotine (panels B, H, N), p-coumaric acid (panels C, I, O) and Mediator (panels D, J, P). In these cases, biofilms appeared as widespread agglomerates of cells (green fluorescence) attached to the membrane surface and embedded in ECM-containing EPS (red fluorescence). On the contrary, the exposure to Reldan (panels E, K, Q) and Roundup (panels F, L, R) affected the biofilm growth. In particular, the exposure to Reldan resulted in thinner biofilm by A. kunkeei BEE4, whereas A. kunkeei BV61 and PL13 just developed small agglomerates of cells with discontinuous EPS production. For A. kunkeei PL13, such agglomerates were also sporadic. The exposure to Roundup almost completely counteracted the development of biofilm by A. kunkeei BEE4 and BV61, while A. kunkeei PL13 developed a thin and discontinuous layer of biofilm characterized by small agglomerates of cells with low EPS production (Fig. 5).

The values of cell density confirmed the previous observations (Fig. 6A). The exposure to Roundup resulted in the lowest (P < 0.05) level in A. kunkeei BEE4 (3.6 ± 0.12 Log CFU g⁻¹), BV61 (4.79 ± 0.05 Log CFU g⁻¹) and PL13 (6.70 ± 0.05 Log CFU g⁻¹) biofilms. Reldan also resulted in a significant (P < 0.05) reduction of cell density for all strains compared to the control. On the contrary, the presence of nicotine or p-coumaric acid did not affect the cell density in A. kunkeei BEE4 and BV61 biofilm, while it resulted in a higher (P < 0.05) cell density for A. kunkeei
PL13 compared to the control (8.5 ± 0.08 and 8.8 ± 0.07 Log CFU g⁻¹ respectively). Mediator caused a significant ($P < 0.05$) decrease of *A. kunkeei* BEE4 cell density, while it did not affect the values in *A. kunkeei* BV61 and PL13 compared to the control.

Control biofilm biomass was 8.81 ± 0.16, 5.98 ± 0.11 and 7.33 ± 1.83 mg for *A. kunkeei* BEE4, BV61 and PL13 respectively (Fig. 6B). For all strains, the exposure to Reldan and Roundup resulted in a significantly ($P < 0.05$) lower biomass compared to control biofilms, with Roundup showing the highest inhibitory effect. Final values under exposure to Roundup were 0.02 ± 0.00, 0.13 ± 0.04, 0.04 ± 0.01 mg for *A. kunkeei* BEE4, BV61 and PL13 respectively. Under exposure to nicotine, *p*-coumaric acid and Mediator, the biomass of *A. kunkeei* BEE4 and PL13 biofilms did not vary significantly ($P > 0.05$) compared to controls. On the contrary, biofilm by *A. kunkeei* BV61 resulted in a significantly ($P < 0.05$) higher biomass after exposure to Mediator and nicotine, reaching values of 7.57 ± 0.19 and 7.48 ± 0.40 mg, respectively, while no differences compared to control were observed under *p*-coumaric acid exposure.

*Characterization of ECM*

Biofilms grown under exposure to different plant secondary metabolites and agrochemicals were further characterized through extraction of ECM and quantification of different matrix components (EPS, proteins and DNA) (Fig. 6C). For all strains, control biofilms and biofilms exposed to agents that did not produce a significant biomass reduction (i.e. nicotine, *p*-coumaric acid and Mediator) had similar relative abundances of ECM components, with EPS being the dominant fraction. The highest abundance of EPS was detected in ECM extracted from biofilm treated with Mediator, *p*-coumaric acid and non-treated biofilm for *A. kunkeei* BEE4, BV61 and PL13 respectively (accounting for ca. 86.1%, 88.2% and 92.5% of total ECM respectively). Proteins were also present in ECM. For *A. kunkeei* BEE4, the highest relative abundance was found in biofilm treated with *p*-coumaric acid (ca. 6.8% of total ECM), while for *A. kunkeei* BV61 and PL13 in biofilm treated with Mediator (ca. 4.4% and 6.4% of total ECM respectively). The ECM of *A. kunkeei* BEE4, BV61 and PL13 biofilms had the highest relative abundance of DNA under treatment with *p*-coumaric acid, nicotine and Mediator respectively (ca. 14.9%, 9.1% and 11.3%). In biofilm by *A. kunkeei* BEE4 exposed to Reldan, the ECM profile revealed similar relative abundances compared to control biofilm and previous treatments, even if the total biomass was significantly lower. Similarly, in *A. kunkeei* BV61 and PL13 biofilm exposed to Reldan, EPS was still the dominant fraction even if *A. kunkeei* BV61 ECM showed higher relative abundance of protein and DNA (ca. 19% and 16% of total ECM respectively) compared to control biofilm and previous treatments, whereas no proteins

![Fig. 3. Cell density (Log CFU g⁻¹) (A) and biomass (mg) (B) of *Apilactobacillus kunkeei* BEE4, BV61 and PL13 biofilms that, after 96 h of growth, were exposed to various plant secondary metabolites (nicotine and *p*-coumaric acid) and agrochemicals (Mediator, Reldan and Roundup). 'Control' indicates the control sample without supplementation of phytosanitary products and plant secondary metabolites. Data are the mean of three separate analyses ± standard deviations. Bars with different superscript letters differ significantly ($P < 0.05$).](image-url)
were detected in \textit{A. kunkeei} PL13 ECM. Differently, ECM of all biofilms showed the presence of only DNA when strains grew under exposure to Roundup.

**Discussion**

Honeybees, living in social colonies, host a core microbiome that influences their diet and immune function (Horak et al., 2020). On the other hand, several recent studies have confirmed that agrochemicals exposure can perturb the gut homeostasis of honeybees and reduce their survival status (Rouzé et al., 2019; Yang et al., 2020; Motta and Moran, 2020; Zhu et al., 2020; Cuesta-Maté et al., 2021). The emerging evidences supporting the beneficial role played by symbiotic fructophilic lactobacilli within the honeybee’s crop (Forsgren et al., 2010; Filannino et al., 2016; Arredondo et al., 2018; Lamei et al., 2019; Daisley et al., 2020b; Iorizzo et al., 2020; Bielik et al., 2021) impose to improve the knowledge about their behaviour if exposed to agrochemicals associated to bees decline, as well as to plant secondary metabolites ingested as part of diet (Filannino et al., 2016).

Six fructophilic lactobacilli belonging to \textit{F. fructosus} and \textit{A. kunkeei} species and previously isolated from honeybee (\textit{Apis mellifer L.}) digestive tract and from bee-collected pollen (Filannino et al., 2016; Di Cagno et al., 2019) were used in this study. After a preliminary screening based on carbon sources utilization and on the capability to produce EPS, which are critical for biofilm formation, the \textit{A. kunkeei} strains were selected for subsequent sensitivity assays to secondary plant metabolites and agrochemicals, both under planktonic and biofilm conditions. Knowledge on susceptibility of potentially symbiotic fructophilic lactobacilli to antimicrobial agents is still limited (Cuesta-Maté et al., 2021; Pachla et al., 2021). Under the condition of our study, the growth of \textit{A. kunkeei} planktonic cells was completely inhibited by Roundup and Reldan, whereas nicotine, \textit{p}-coumaric acid and Mediator induced the same planktonic pattern of control condition. According to the literature, Roundup susceptibility differed amongst microorganisms (Bonnet et al., 2007; Hernando et al., 2007) and depends on the presence of a 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme of class I (sensitive to glyphosate) or class II (insensitive to glyphosate) in the shikimate pathway (Motta and Moran, 2020). Findings obtained after exposure to Roundup are in agreement with previous studies reporting that the majority of beneficial bacteria, including \textit{Enterococcus faecalis}, \textit{Enterococcus faecium}, \textit{Bacillus badius}, \textit{Bifidobacterium adolescentis} and \textit{Lactobacillus} spp., were shown to be from moderately to extremely sensitive to Roundup (Shehata et al., 2013). Furthermore, the exposure to glyphosate severely disrupted bees’ beneficial gut microbiome, reducing bee health and pollination efficacy (Motta and Moran, 2020). However, different molecular mechanisms underlying glyphosate resistance in bacteria were also highlighted demonstrating the adaptation capacity of some species thanks to genomic alterations, although A.
kunkeei does not appear in such list. The mechanisms included an increased production of EPSPS, the degradation or detoxification of the herbicide, and the uptake decrease combined with an export increase of such agrochemical (Hertel et al., 2021). Reldan, an organophosphorus insecticide, having a broad range of actions is used to eradicate pests from agricultural and farming regions (Nita-Lazar et al., 2016). Its active ingredient (chlorpyrifos-methyl) is an acetylcholinesterase inhibitor. As cholinesterase activity (Goldstein and Goldstein, 1953; Rochu et al., 1998) and acetylcholinesterase enzymes (Sánchez et al., 2012; Pham et al., 2020) were reported in bacteria, it could be hypothesized that the effect of chlorpyrifos-methyl on A. kunkeei is mediated by its canonical mechanism of action. However, studies through genomics approaches should be performed to clarify its mechanism on this target organism. In agreement with our finding, Yang et al. (2019) noted that honeybee gut microbiome diversity declined between 15 and 30 days after exposure to chlorpyrifos-methyl. However, also the biodegradation of Reldan by microorganisms has been widely reported (Digrak et al., 1995; Abd El-Mongy and Abd El-Ghany, 2009), either through a rapid bacterial defence response by metabolizing the insecticides (Nita-Lazar et al., 2016) or acquired resistance by genetic mutations (Martinez and Baquero, 2000). Consequently, the potential effects of such resistance development on physiology, ecology and evolution of A. kunkeei are additional new interesting questions to be investigated to fully evaluate the overall range of

---

Fig. 5. Confocal microscopy analysis of biofilms produced by *Apilactobacillus kunkeei* BEE4 (panels A–F), BV61 (G–L) and PL13 (M–R) under exposure to nicotine (panels B, H, N), p-coumaric acid (C, I, O), Mediator (D, J, P), Reldan (E, K, Q) and Roundup (F, L, R). Panels A, G, M represent control biofilms without exposure to agrochemicals or plant secondary metabolites. Bacterial cells show green fluorescence and EPS in biofilm ECM show red fluorescence. Scale bars represent 50 μm; units of x, y and z axes are μm.

Fig. 6. Cell density (Log CFU g⁻¹) (A), biomass (mg) (B) and relative abundance of different extracellular polymeric matrix components (C) of *Apilactobacillus kunkeei* BEE4, BV61 and PL13 biofilms after 96 h of growth under exposure to various plant secondary metabolites (nicotine and p-coumaric acid) and agrochemicals (Mediator, Reldan and Roundup). ‘Control’ indicates the control sample without supplementation of phytosanitary products and plant secondary metabolites. Data are the mean of three separate analyses ± standard deviations. Bars with different superscript letters differ significantly (P < 0.05).
consequences for honeybees due to the exposure to this agrochemical. On the other hand, our observations on the effects of Mediator are only partially confirmed by literature data, which are somewhat controversial. Although a substantial reduction in the abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. in honeybees gut was reported by some studies (Rouzé et al., 2019), other studies claimed that the oral exposure does not affect the community structure of gut microbiome (Gain, 2018).

Once established the sensibility of *A. kunkeei* planktonic cells to Roundup and Reldan, we focused the further experiments on the effects of plant secondary metabolites and agrochemicals on sessile growth of *A. kunkeei*, since fructophilic lactobacilli mostly inhabit the crop of honeybee as biofilm communities (Vásquez et al., 2012). Biofilm defined as a microbial community attached to a surface and embedded in ECM is consisting of EPS, proteins and DNA (Polo et al., 2011). Microbial cells within a biofilm usually display an increased resistance to stress conditions and antimicrobial agents compared to their planktonic counterpart (Mah and O’Toole, 2001; Sandasi et al., 2010; Polo et al., 2011; Jadhav et al., 2013). Moreover, biofilm may serve as reservoirs of beneficial bacteria, in which these microorganisms can overcome stress conditions and exert their positive role, such as preventing pathogens from accessing host epithelial cells (Silizová et al., 2015; Berrios et al., 2018; Horak et al., 2020). Although the relationship between the honeybee immune system and gut symbionts is not well defined, different microbial species harbour distinct functional capacities attributed to host interaction and biofilm development (Engel et al., 2012). We found that cells of *A. kunkeei* in mature biofilms display higher tolerance to Reldan and Roundup compared to the planktonic cells, whereas growing biofilms did not guarantee the same protective effect (Polo et al., 2011). Growing biofilms represent more vulnerable systems than mature biofilms, most likely due to the lack of an abundant ECM barrier able to protect against active ingredients penetration, as observed by CLSM. The ECM represents a barrier that protects cells from the penetration of active ingredients (Mah and O’Toole, 2001). In addition, in the earlier stage of biofilm growth, other resistance mechanisms could be not well established yet. Another noteworthy finding is the level of susceptibility to the three agrochemicals that resulted to be strain-specific. Focusing on the secondary plant metabolites, surprisingly they had had a growth-promoting effect on *A. kunkeei* cells in sessile communities, although no effect was found on planktonic growth. Nicotine is known to exert multiple favourable and unfavourable physiological functions on honeybees (Gui et al., 2021). It was shown that amounts naturally occurring in nectar did not impact hatching success or larval survival, but greater nicotine concentrations revealed detrimental effects (Singaravelan et al., 2006). On the contrary, little is known about its impact on bee microbiota. Recently, the effect on the human gut microbiota is a heavily debated topic. Nicotine boosts the Bacteroidetes’ phylum while it decreases the Firmicutes and Proteobacteria phyla (Lee et al., 2018). p-Coumaric acid is found ubiquitously in pollen and was associated to increased longevity of honeybees (Wong et al., 2018), and recent studies reported that it can enhance the richness and abundance of honeybee gut microbiota when added as dietary supplementation (Geidert et al., 2021). Whether the antibacterial activity of phenolic acids is known, lactic acid bacteria have a good ability to tolerate them, albeit this capacity is species-specific, and it is conceivably a benefit for bacteria from phenolic acid metabolism (Filannino et al., 2014, 2016). For example, it has been suggested that heterofermentative lactic acid bacteria, which include fructophilic lactobacilli, may use phenolic acid as additional electron acceptors and gain extra metabolic energy to counteract stressful conditions (Filannino et al., 2014, 2016). As content and composition of ECM represent key elements that strongly influences biofilm structure and properties (Limoli et al., 2015; Di Martino, 2018; George and Halami, 2019), the relative abundance of its main components was analysed to investigate potential changes due to plant secondary metabolites and agrochemicals. This included the determination the EPS, proteins and DNA content of the biofilm matrix, studying relationship with exposition to active agents. EPS resulted in the main components of ECM both with and without exposure to Mediator, Reldan and plant secondary metabolites. This finding is in agreement with other studies on lactic acid bacteria biofilm that highlighted the protective role of EPS from adverse factors (Nguyen et al., 2020). However, to the best of our knowledge, no data exist in literature about the composition of ECM in fructophilic lactobacilli biofilm and relative abundance of matrix components. Although both Reldan and Roundup hindered the biofilm formation, only the second caused a clear inhibition of ECM production, with DNA being the only detected component after exposure. It could explain the strong contrasting action towards the biofilm growth, since both EPS and proteins play an essential role in biofilm expansion other than as protective agents (Karygianni et al., 2020). Also, extracellular DNA aids in bacterial attachment and aggregation, but it is critical especially in the early stages of biofilm formation (Panlilio and Rice, 2021).

**Conclusion**

We concluded that *A. kunkeei* cells embedded in biofilms have increased resistance to chlorpyrifos-methyl...
and glyphosate, which instead strongly threaten planktonic cells. However, sessile communities are more susceptible if the exposure happens at the preliminary steps of biofilm formation, when such agrochemicals still exert an inhibitory/control effect. Our results lead to re-evaluate the potential symbiotic role of A. kunkeei in established sessile communities, even under environmental stress conditions due to the exposure to agrochemicals. Definitely, further studies about the mechanisms of action underlying the observed effects by tested agrochemicals must be performed, and mechanisms of resistance displayed by target strains need to be examined in depth through genomics approaches in order to reach a holistic understanding of such findings.

Expanding knowledge on adaptation strategies pursued by honeybees-associated microbial communities offers new opportunity for creation of effective tools to preserve the health of honeybees. The strain-specific abilities to resist agrochemicals and to form biofilms can be considered useful phenotypes during the selection of probiotic preparations for honeybees. Furthermore, the growth-promoting effect exerted by nicotine and p-coumaric acid in bacterial sessile communities may prove useful for defining dietary interventions aimed at keeping eubiosis status in honeybees-associated microbiota.

**Experimental procedures**

**Microorganisms and culture conditions**

Six fructophilic lactobacilli, belonging to the Culture Collection of the Department of Soil, Plant and Food Science, University of Bari Aldo Moro (Bari, Italy), were used in this study (Table S1). All strains were previously isolated from *Apis mellifera*'s gut and bee-collected pollen and belong to *F. fructosus* and *A. kunkeei* species. Strains were cultured on Fructose Yeast extract Peptone (FYP) at 30°C for 16-20 h as described by Filannino *et al.* (2016) and identified by partial sequencing of the 16S rRNA.

**Kinetic of growth and carbon source utilization**

Fructophilic lactobacilli were propagated until the late exponential (LE) growth phase was reached (Filannino *et al.*, 2019). Then, cells were harvested by centrifugation (10,000 rpm, 10 min at 4°C), washed twice in 50 mM of sterile potassium phosphate buffer (pH 7.0) and singly inoculated in FYP broth medium (containing fructose as main carbon source) and in modified FYP (mFYP) broth containing 10 g l⁻¹ of sucrose instead of fructose (sucrose as main carbon source) to a final cell density of ca. 7.0 log CFU ml⁻¹. FYP and mFYP were used as substrates to investigate the kinetic growth and carbohydrates metabolism in fructophilic lactobacilli strains. Cultures were incubated at 30°C for 24 h and the kinetics of growth monitored by measuring the turbidity at 620 nm (Ab620). Kinetics of growth was determined and modelled according to the Gompertz equation as modified by Zwietering *et al.* (1990): \( y = k + A \exp(-\exp([\mu_{\text{max}}/A](\lambda - t) + 1)) \). When the Ab620 was the dependent variable to be modelled, \( k \) was the initial level of Ab620 units, \( A \) was the difference in Ab620 units between inoculation and the stationary phase, \( \mu_{\text{max}} \) was the maximum growth rate (Ab620 units h⁻¹), \( \lambda \) was the length of the lag phase (h) and \( t \) was the time (h). Data were fitted using non-linear regression procedure of the *Statistica* 7.0 software (Statsoft, Tulsa, OK, USA).

The concentrations of fructose and sucrose in FYP and mFYP cultures were determined through an HPLC (High Performance Liquid Chromatography) system Ultimate 3000 (Dionex, Germering, Germany), equipped with a Spherisorb column (Waters, Millford, CT, USA) and a Perkin Elmer 200a refractive index detector, to determine the utilization of main carbon sources. Peaks were identified by comparing elution times and spiking samples with known quantities of standard solutions of fructose and sucrose (Rizzello *et al.*, 2010).

**Screening for the production of exopolysaccharides**

Fructophilic lactobacilli were plated on FYP and mFYP agar for a screening based on their ability to produce EPS and form mucous colonies. EPS production was assessed through visual inspection of colonies. The level of EPS production of fructophilic lactobacilli was also quantified in FYP and mFYP broth as described previously by Jin *et al.* (2019) with slight modifications. Briefly, after 24 h of cell growth at 30°C, the culture was boiled for 15 min and then treated with 17% (v/v) of trichloroacetic acid (85%) for 2 h. Cells were pelleted at 18,000 g for 25 min. Five milliliters aliquot of the supernatant were incubated overnight at 4°C with 5 volumes of 95% ethanol (-20°C) before centrifugation (18,000 g for 20 min) to collect the precipitates. Crude EPS precipitants were re-dissolved in 5 ml of demineralized water, dialyzed using a commercially available dialysis bag (12–14 kDa) at 4°C for 48 h, freeze dried at least 24 h (Epsilon 2-6D LSC plus freeze-drier, Martin Christ, Osterode am Harz, Germany) and finally weighed.

**Plant secondary metabolites and agrochemicals**

Two plant secondary metabolites (p-coumaric acid and nicotine), and three agrochemicals with herbicide (glyphosate) and insecticides (imidacloprid and chlorpyrifos-methyl) actions commonly used for agronomical practices were selected for the study and analysed individually. Table 2 reports the list with references.
Survival test on planktonic cells (as reported by manufacturer). Concentrations are commonly used for agronomical treatments separately for each chemical. Ab620 was measured metabolites or agrochemicals. The test was performed FYP broth was supplemented with plant secondary throughout the incubation at 30°C. Diluted culture was used to inoculate individual sterile, overnight cultures of fructophilic lactobacilli strains were diluted to a turbidity (600 nm) of 0.20 in 50 mM of sterile potassium phosphate buffer (pH 7.0). One 5-fold diluted culture was used to inoculate individual sterile, Whatman, Maidstone, UK) resting on FYP agar medium (control) or on FYP agar supplemented with examined agents, and incubated for 96 h. In both cases, the plates were inverted after inoculum and incubated at 30°C, with the membrane-supported biofilms transferred to fresh culture medium every 10–12 h (Anderl et al., 2000). Seven membranes were prepared for each experiment. After incubation, membrane-supported biofilms were visually inspected and photographed. Three membrane-supported biofilms were weighed with analytical balance (Praetum 124-1S; Sartorius Lab Instruments, Gottingen, Germany) to quantify the biomass. Then, each one was transferred in 10 ml of potassium phosphate buffer, cells were detached and suspended through vortex (1 min at maximum speed), and serially diluted. Dilutions were plated on FYP agar medium and CFU were enumerated.

Confocal laser scanning microscopy analysis
One membrane-supported biofilm for each experiment was visually inspected and photographed by CLSM (Leica SP8LIA; Leica Microsystems, Bucinasco, Italy). Biofilms were carefully mounted on glass slides. Bacterial cells and polysaccharides of extracellular polymeric matrix (ECM) were stained with a 15 µM of SYTO9 (Invitrogen, Eugene, OR, USA) and 200 µg ml⁻¹ of texas red-labelled Concanavalin A (ConA, Invitrogen, stock solution, 5 mg ml⁻¹ in 0.1 M sodium bicarbonate) solution in PBS (pH 7.5). Samples were incubated under dark conditions for 30 min at room temperature. Then, samples were visualized by using 488- and 552-nm lasers. Fluorescence emission was observed between 500–565 nm (for FDA) and 565–645 nm (for ConA). Images were captured with a 40× oil immersion objective, and analysed with the software LAS X.

Structure of biofilm ECM
ECM was extracted from three membrane-supported biofilms according to the method described by Chiba et al. (2015). Specifically, the colony biofilms were scraped

Table 2. Plant secondary metabolites and agrochemicals (active ingredient, form and concentration) used in the study.

| Plant secondary metabolites | Form         | Concentration | References                  |
|----------------------------|--------------|---------------|-----------------------------|
| Nicotine                   | Pure compound | 30 µM         | Köhler et al. (2012)        |
| p-Coumaric acid            | Pure compound | 0.5 mM        | Wong et al. (2018)          |
| Imidacloprid               | Mediator Top | 0.6 ml l⁻¹    | Rouzé et al. (2019)         |
| Chlorpyrifos-methyl        | Reldan™ 22   | 2.0 ml l⁻¹    | Favaro et al. (2019)        |
| Glyphosate                 | Roundup® Power 2.0 | 3.0 ml l⁻¹ | Motta and Moran (2020)      |

and nicotine were purchased from Sigma-Aldrich and analysed in aqueous solutions at 0.5 mM and 30 µM respectively. These concentrations were chosen as they are within the range typically encountered by adult honeybees (Köhler et al., 2012; Wong et al., 2018). Glyphosate, imidacloprid and chlorpyrifos-methyl were examined through the commercially available agrochemical products Roundup® Power 2.0 (Monsanto, Milan, Italy), Mediator Top (Massó Agro Department, Cinisello Balsamo, Italy) and Reldan™ 22 (Corteva Agroscience, Milan, Italy), respectively, which contain such compounds as active ingredients. These products were selected as they are commonly adopted for agronomical practices and were considered harmful for honeybee or associated to effects on honeybee gut microbiota (Favaro et al., 2019; Rouzé et al., 2019; Motta and Moran, 2020). Analysed concentrations were 3, 0.6 and 2 ml l⁻¹ respectively. Such concentrations were commonly used for agronomical treatments (as reported by manufacturer).

Survival test on planktonic cells
A cell suspension of each strain (ca. 7 Log CFU l⁻¹) in FYP broth was supplemented with plant secondary metabolites or agrochemicals. The test was performed separately for each chemical. Ab620 was measured throughout the incubation at 30°C to monitor the bacterial growth. Data were modelled to determine the kinetics of growth as described in the previous section.

Biofilm preparation and exposure tests
Overnight cultures of fructophilic lactobacilli strains were diluted to a turbidity (600 nm) of 0.20 in 50 mM of sterile potassium phosphate buffer (pH 7.0). One 5-µl drop of diluted culture was used to inoculate individual sterile, black, polycarbonate membrane filters (diameter, 2.5 cm; pore size, 0.2 µm; Whatman, Maidstone, UK) resting on FYP agar medium (control) or on FYP agar supplemented with plant secondary metabolites or agrochemical treatments (Table 2). The membranes were sterilized by UV exposure (15 min per side) before inoculation. The effect of exposure was assessed both on already developed biofilm and during biofilm growth. In the first case, a first incubation period (96 h) occurred on FYP agar without any supplementation to allow the biofilm growth. Then, a second incubation period (96 h) occurred on FYP agar supplemented with plant secondary metabolites or agrochemicals. In the second case, the culture of biofilms was carried out directly on FYP agar supplemented with examined agents, and incubated for 96 h. In both cases, the plates were inverted after inoculum and incubated at 30°C, with the membrane-supported biofilms transferred to fresh culture medium every 10–12 h (Anderl et al., 2000). Seven membranes were prepared for each experiment.

After incubation, membrane-supported biofilms were visually inspected and photographed. Three membrane-supported biofilms were weighed with analytical balance (Praetum 124-1S; Sartorius Lab Instruments, Gottingen, Germany) to quantify the biomass. Then, each one was transferred in 10 ml of potassium phosphate buffer, cells were detached and suspended through vortex (1 min at maximum speed), and serially diluted. Dilutions were plated on FYP agar medium and CFU were enumerated.

Confocal laser scanning microscopy analysis
One membrane-supported biofilm for each experiment was visually inspected and photographed by CLSM (Leica SP8LIA; Leica Microsystems, Bucinasco, Italy). Biofilms were carefully mounted on glass slides. Bacterial cells and polysaccharides of extracellular polymeric matrix (ECM) were stained with a 15 µM of SYTO9 (Invitrogen, Eugene, OR, USA) and 200 µg ml⁻¹ of texas red-labelled Concanavalin A (ConA, Invitrogen, stock solution, 5 mg ml⁻¹ in 0.1 M sodium bicarbonate) solution in PBS (pH 7.5). Samples were incubated under dark conditions for 30 min at room temperature. Then, samples were visualized by using 488- and 552-nm lasers. Fluorescence emission was observed between 500–565 nm (for FDA) and 565–645 nm (for ConA). Images were captured with a 40× oil immersion objective, and analysed with the software LAS X.

Structure of biofilm ECM
ECM was extracted from three membrane-supported biofilms according to the method described by Chiba et al. (2015). Specifically, the colony biofilms were scraped

© 2022 The Authors. Microbial Biotechnology published by Society for Applied Microbiology and John Wiley & Sons Ltd., Microbial Biotechnology, 15, 2160–2175
with a scraper and suspended with 1 ml of 1.5 M potassium phosphate buffer solution. The suspensions were centrifuged at 5000 g for 10 min at 25°C without any incubation period. The supernatants were collected as ECM fractions.

The concentration of DNA in ECM fractions was measured with NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of protein was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). The concentration of protein was measured using a Bradford protein assay kit (Bio-Rad, Waltham, MA, USA). The concentration of protein was measured using a Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). The concentration of protein was measured using a Bradford protein assay kit (Bio-Rad, Waltham, MA, USA).

Statistical analysis

All experiments were carried out considering three experimental replicates and three analytical replicates. Data were submitted to analysis of variance by the General Linear Model (GLM) of R statistical package (R, version 1.6.2 rcompanion.org/handbook/). Pairwise comparison of treatment means was achieved by Tukey-adjusted comparison procedure with \( P \) value (\( P < 0.05 \) (Mangiafico, 2016). Clustering analysis, using the default method available in R and based on Euclidean distance and McQuitty linkage, was performed.

Acknowledgements

This work was supported by the Faculty of Science and Technology of the Free University of Bozen-Bolzano and the Open Access Publishing Fund of the Free University of Bozen-Bolzano. The authors thank EFRE FESR Südtirol Alto Adige – Micro4Food. The authors thank Dr. Sara Casagrande for her help throughout this research.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Abd El-Mongy, M., and Abd El-Ghany, T.M. (2009) Field and laboratory studies for evaluating the toxicity of the insecticide Reldan on soil fungi. Int Biodeterior Biodegradation 63: 383–388.

Aizen, M.A., and Harder, L.D. (2009) The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr Biol 19: 915–918.

Anderl, J.N., Franklin, M.J., and Stewart, P.S. (2000) Role of antibiotic penetration limitation in Klebsiella pneumoniae biofilm resistance to ampicillin and ciprofloxacin. Antimicrob Agents Chemother 44: 1818–1824.

Anderson, K.E., and Ricigliano, V.A. (2017) Honey bee gut dysbiosis: a novel context of disease ecology. Curr Opin Insect Sci 22: 125–132.

Anderson, K.E., Sheehan, T.H., Eckholm, B.J., Mott, B.M., and DeGrandi-Hoffman, G. (2011) An emerging paradigm of colony health: microbial balance of the honey bee and hive (Apis mellifera). Insect Soc 58: 431–444.

Anderson, K.E., Sheehan, T.H., Mott, B.M., Maes, P., Snyder, L., Schwan, M.R., et al. (2013) Microbial ecology of the hive and pollination landscape: bacterial associates from floral nectar, the alimentary tract and stored food of honey bees (Apis mellifera). PLoS One 8: e83125.

Arredondo, D., Castelli, L., Porini, M.P., Garrido, P.M., Eguras, M.J., Zunino, P., and Antunez, K. (2018) Lactobacillus kunkeei strains decreased the infection by honey bee pathogens Paenibacillus larvae and Nosema ceranae. Benef Microbes 9: 279–290.

Baffoni, L., Gaggia, F., Alberoni, D., Cabbri, R., Nanetti, A., Biavati, B., and Di Gioia, D. (2016) Effect of dietary supplement of Bifidobacterium and Lactobacillus strains in Apis mellifera L. against Nosema ceranae. Benef Microbes 7: 45–51.

Berrios, P., Fuentes, J.A., Salas, D., Carrero, A., Aldea, P., Fernandez, F., and Trombert, A.N. (2018) Inhibitory effect of biofilm forming Lactobacillus kunkeei strains against virulent Pseudomonas aeruginosa in vitro and in honeycomb moth (Galleria mellonella) infection model. Benef Microbes 9: 257–268.

Bieli, B., Molnár, L., Vrabec, V., Andrásová, R., Maruščáková, I.C., Nemcová, R., et al. (2021) Biofilm forming lactic acid bacteria of honey bee origin intended for potential probiotic use. Acta Vet Hung 68: 345–353.

Bonnet, J.L., Bonnemoy, F., Dusser, M., and Bohatier, J. (2007) Assessment of the potential toxicity of herbicides and their degradation products to nontarget cells using two microorganisms, the bacteria Vibrio fischeri and the ciliate Tetrahymena pyriformis. Environ Toxicol 162: 78–91.

Brodsher, G., Gray, A., Adlajane, N., Bellis, A., Bruhzards, V., Chamière, J.D., and Danihl, J. (2018) Multi-country loss rates of honey bee colonies during winter 2016/2017 from the COLOSS survey. J Apic Res 57: 452–457.

Chiba, A., Sugimoto, S., Sato, F., Hori, S., and Mizunoe, Y. (2015) A refined technique for extraction of extracellular matrices from bacterial biofilms and its applicability. Microb Biotechnol 8: 392–403.

Corby-Harris, V., Maes, P., and Anderson, K.E. (2014) The bacterial communities associated with honey bee (Apis mellifera) foragers. PLoS One 9: e95056.

Cox-Foster, D.L., Conlon, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., and Lipkin, W.I. (2007) A metagenomic survey of microbes in honeybee colony collapse disorder. Science 318: 283–287.
Cuesta-Maté, A., Renelies-Hamilton, J., Kryger, P., Jensen, A.B., Sinotte, V.M., and Poulsen, M. (2021) Resistance and vulnerability of honeybee (Apis mellifera) gut bacteria to commonly used pesticides. Front Microbiol 12: 2428.

Daisley, B.A., Chmiel, J.A., Pitek, A.P., Thompson, G.J., and Reid, G. (2020a) Missing microbes in bees: How systematic depletion of key symbionts erodes immunity. Trends Microbiol 28: 1010–1021.

Daisley, B.A., Pitek, A.P., Chmiel, J.A., Al, K.F., Chernyshova, A.M., Faragalla, K.M., et al. (2020b) Novel probiotic approach to counter Paenibacillus larvae infection in honey bees. ISME J 14: 476–491.

Di Cagno, R., Filannino, P., Cantatore, V., and Gobbetti, M. (2019) Novel solid-state fermentation of bee-collected pollen emulating the natural fermentation process of bee bread. Food Microbiol 82: 218–230.

Di Martino, P. (2018) Extracellular polymeric substances, a key element in understanding biofilm phenotype. AIMS Microbiol 4: 274.

Digrak, M., Ozcelik, S., and Celik, S. (1995) Degradation of ethion and methidathion by some microorganisms. In 35th IUPAC Congress, Vol. 14, pp. 19–84.

Engel, P., Martinson, V.G., and Moran, N.A. (2012) Functional diversity within the simple gut microbiota of the honey bee. Proc Natl Acad Sci USA 109: 11002–11007.

Favaro, R., Bauer, L.M., Rossi, M., D’Ambrosio, L., Bücher, E., and Angell, S. (2019) Botanical origin of pesticide residues in pollen loads collected by honeybees during and after apple bloom. Front Physiol 10: 1069.

Filannino, P., Di Cagno, R., Addante, R., Pontonio, E., and Gobbetti, M. (2016) Metabolism of fructophilic lactic acid bacteria isolated from the Apis mellifera L. bee gut: Phenolic acids as external electron acceptors. Appl Environ Microbiol 82: 6899–6911.

Filannino, P., Di Cagno, R., Tlais, A.Z.A., Cantatore, V., and Gobbetti, M. (2019) Fructose-rich niches traced the evolution of lactic acid bacteria toward fructophilic species. Crit Rev Microbiol 45: 65–81.

Filannino, P., Gobbetti, M., De Angelis, M., and Di Cagno, R. (2014) Hydroxycinnamic acids used as external acceptors of electrons: an energetic advantage for strictly heterofermentative lactic acid bacteria. Appl Environ Microbiol 80: 7574–7582.

Forsgren, E., Olofsson, T.C., Vásquez, A., and Fries, I. (2010) Novel lactic acid bacteria inhibiting Paenibacillus larvae in honey bee larvae. Apidologie 41: 99–108.

Gain, E.G. (2018) A metagenomic analysis of the honey bee gut microbiome following oral imidacloprid exposure, Doctoral dissertation, Middle Tennessee State University.

Geldert, C., Abdo, Z., Stewart, J.E., and Arathi, H.S. (2021) Dietary supplementation with phytochemicals improves diversity and abundance of honey bee gut microbiota. J Appl Microbiol 130: 1705–1720.

George, J., and Halami, P.M. (2019) Presence of extracellular DNA & protein in biofilm formation by gentamicin-resistant Lactobacillus plantarum. Indian J Med Res 149: 257.

Goldstein, D.B., and Goldstein, A. (1953) An adaptive bacterial cholinesterase from a Pseudomonas species. J Gen Microbiol 8: 8–17.

Gui, X., Yang, Z., and Li, M.D. (2021) Effect of cigarette smoke on gut microbiota: state of knowledge. Front Physiol 12: 816.

Hamdi, C., Balloi, A., Essanaa, J., Crotti, E., Gonella, E., Raddadi, N., et al. (2011) Gut microbiome dysbiosis and honeybee health. J Appl Entomol 135: 524–533.

Hernando, M.D., De Vettori, S., Bueno, M.M., and Fernández-Alba, A.R. (2007) Toxicity evaluation with Vibrio fisheri test of organic chemicals used in aquaculture. Chemosphere 68: 724–730.

Hertel, R., Gibhardt, J., Martiensen, M., Kuhn, R., and Commichau, F.M. (2021) Molecular mechanisms underlying glyphosate resistance in bacteria. Environ Microbiol 23: 2891–2905.

Horak, R.D., Leonard, S.P., and Moran, N.A. (2020) Symbionts shape host innate immunity in honeybees. Proc R Soc B Biol Sci 287: 20201184.

Hristov, P., Neov, B., Shumkova, R., and Palova, N. (2020a) Significance of apoidea as main pollinators. ecological and economic impact and implications for human nutrition. Diversity 12: 280.

Hristov, P., Shumkova, R., Palova, N., and Neov, B. (2020b) Factors associated with honey bee colony losses: a mini-review. Vet Sci 7: 166.

Iorizzo, M., Lombardi, S.J., Ganassi, S., Testa, B., Ianio, M., Letizia, F., et al. (2020) Antagonistic activity against Ascosphaera apis and functional properties of Lactobacillus kunkeei strains. Antibiotics 9: 262.

Jadhav, S., Shah, R., Bhave, M., and Palombo, E.A. (2013) Inhibitory activity of yarrow essential oil on Listeria monocytogenes and biofilms. Food Control 29: 125–130.

Jin, H., Jeong, Y., Yoo, S.H., Johnston, T.V., Ku, S., and Ji, G.E. (2019) Isolation and characterization of high exopolysaccharide-producing Weissella confusa VP30 from young children’s feces. Microb Cell Factor 18: 1–13.

Karygianni, L., Ren, Z., Koo, H., and Thurnheer, T. (2020) Biofilm matrixome: extracellular components in structured microbial communities. Trends Microbiol 28: 668–681.

Köhler, A., Pirk, C.W., and Nicolson, S.W. (2012) Honeybees and nectar nicotine: deterrence and reduced survival versus potential health benefits. J Insect Physiol 58: 286–292.

Kohlmeyer, P., Holländer, K., and Meunier, J. (2016) Survival after pathogen exposure in group-living insects: don’t forget the stress of social isolation! J Evol Biol 29: 1867–1872.

Lamei, S., Stephan, J.G., Riesbeck, K., Vásquez, A., Olofsson, T., Nilson, B., et al. (2019) The secretome of honey bee-specific lactic acid bacteria inhibits Paenibacillus larvae growth. J Apic Res 58: 405–412.

Lee, H.-W., Park, S.-H., Weng, M.-W., Wang, H.-T., Huang, W.C., Lepor, H., et al. (2018) E-cigarette smoke damages DNA and reduces repair activity in mouse lung, heart, and bladder as well as in human lung and bladder cells. Proc Natl Acad Sci USA 115: 1560–1569.

Limoli, D.H., Jones, C.J., and Wozniak, D.J. (2015) Bacterial extracellular polysaccharides in biofilm formation and function. Microbiol Spectr 3: 3.

Liu, Y.J., Qiao, N.H., Diao, Q.Y., Jing, Z., Vukanti, R., Dai, P.L., and Ge, Y. (2020) Thiacloprid exposure perturbs the gut microbiota and reduces the survival status in honeybees. J Hazard Mater 389: 121818.

Maddaloni, M., Hoffman, C., and Pascual, D.W. (2014) Paratransgenesis feasibility in the honeybee (Apis mellifera) kunkeei biofilm as an extra gear 2173
Panlilio, H., and Rice, C.V. (2021) The role of extracellular factors in the food source of honey bee (Apis mellifera) using Fructobacillus fructosus as a probiotic. J Appl Microbiol 120: 1572–1584.

Mah, T.F.C., and O’Toole, G.A. (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9: 34–39.

Mangialfico, S.S. (2016) Summary and analysis of extension program evaluation in R. version 1.15. 0. rcompanion.org/handbook. Pdf version: rcompanion.org/documents/RHandbookProgramEvaluation.pdf

Manson, J.M., Hancock, L.E., and Gilmore, M.S. (2010) Mechanism of chromosomal transfer of Enterococcus faecalis pathogenicity island, capsule, antimicrobial resistance, and other traits. Proc Natl Acad Sci USA 107: 12269–12274.

Martínez, J.L., and Baquero, F. (2000) Mutation frequencies and antibiotic resistance. Antimicrob Agents Chemother 44: 1771–1777.

Motta, E.V., and Moran, N.A. (2020) Impact of glyphosate on the honey bee gut microbiota: effects of intensity, duration, and timing of exposure. mSystems 5: e00268-20.

Nguyen, H.T., Nguyen, T.H., and Otto, M. (2020) The staphylococcal exopolsaccharide PIA-Biosynthesis and role in biofilm formation, colonization, and infection. Comput Struct Biotechnol J 18: 3324–3334.

Nita-Lazar, M., Galaon, T., Banciu, A., Paun, I., Stoica, C., and Lucaciu, I. (2016) Screening of various harmful compounds in a new bacterial biological model. J Environ Prot Ecol 17: 237–247.

Pachla, A., Ptaszynska, A.A., Wicha, M., Kunat, M., Wydrych, J., Oleška, E., and Malek, W. (2021) Insight into probiotic properties of lactic acid bacterial endosymbionts of Apis mellifera L. derived from the Polish apiary. Saudi J Biol Sci 28: 1890–1899.

Panillo, H., and Rice, C.V. (2021) The role of extracellular DNA in the formation, architecture, stability, and treatment of bacterial biofilms. Biotechnol Bioeng 118: 2129–2141.

Paris, L., Peghaire, E., Mone, A., Diogon, M., Debros, D., Delbac, F., and El Alaoui, H. (2020) Honeybee gut microbiota dysbiosis in pesticide/parasite co-exposures is mainly induced by Nosema ceranae. J Invertebr Pathol 172: 107348.

Pham, V.D., To, T.A., Gagné-Thivierge, C., Couture, M., Lagué, P., Yao, D., et al. (2020) Structural insights into the putative bacterial acetylcholinesterase ChoE and its substrate inhibition mechanism. J Biol Chem 295: 8708–8724.

Polo, A., Diamanti, M.V., Bjarnsholt, T., Haiby, N., Villa, F., Pedeferrì, M.P., and Cappitelli, F. (2011) Effects of photo-activated titanium dioxide nanopowders and coating on planktonic and biofilm growth of Pseudomonas aeruginosa. Photochem Photobiol Phototol 87: 1387–1394.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W.E. (2010) Global pollinator declines: trends, impacts and drivers. Trends Ecol Evol 25: 345–353.

Rangberg, A., Diep, D.B., Rudi, K., and Amdam, G.V. (2012) Paratransgenesis: an approach to improve colony health and molecular insight in honey bees (Apis mellifera)? Integr Comp Biol 52: 89–99.

Rangberg, A., Mathiesen, G., Amdam, G.V., and Diep, D.B. (2015) The paratransgenic potential of Lactobacillus kunkeei in the honey bee Apis mellifera. Benef Microbes 6: 513–523.

Rizzello, C.G., Nionelli, L., Coda, R., De Angelis, M., and Gobbetti, M. (2010) Effect of sourdough fermentation on stabilisation, and chemical and nutritional characteristics of wheat germ. Food Chem 119: 1079–1089.

Rochu, D., Rothlisberger, C., Taupin, C., Renault, F., Gagnon, J., and Masson, P. (1998) Purification, molecular characterization and catalytic properties of a Pseudomonas fluorescens enzyme having cholinesterase-like activity. Biochim Biophys Acta 1385: 126–138.

Rouzé, R., Moné, A., Delbac, F., Belzunces, L., and Blot, N. (2019) The honeybee gut microbiota is altered after chronic exposure to different families of insecticides and infection by Nosema ceranae. Microbes Environ 34: 226–233.

Sánchez, D.G., Otero, L.H., Hernández, C.M., Serra, A.L., Encarnación, S., Domenech, C.E., and Lisa, A.T. (2012) A Pseudomonas aeruginosa PA01 acetylcholinesterase is encoded by the PA4921 gene and belongs to the SGNH hydrolase family. Microbiol Res 167: 317–325.

Sandasi, M., Leonard, C.M., and Viljoen, A.M. (2010) The in vitro antibiofilm activity of selected culinary herbs and medicinal plants against Listeria monocytogenes. Lett Appl Microbiol 50: 30–35.

Shehata, A.A., Schrodl, W., Aldin, A.A., Hafez, H.M., and Kruger, M. (2013) The effect of glyphosate on potential pathogens and beneficial members of poultry microbiota in vitro. Curr Microbiol 66: 350–358.

Singaravelan, N., Inbar, M., Ne’eman, G., Distl, M., Wink, M., and Izhaki, I. (2006) The effects of nectar–nicotine on colony fitness of caged honeybees. J Chem Ecol 32: 49–59.

Sližová, M., Nemcová, R., Maďar, M., Hadroyová, J., Gancarciková, S., Popper, M., and Pistl, J. (2015) Analysis of biofilm formation by intestinal lactobacilli. Can J Microbiol 61: 437–446.

Vásquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekeley, L., and Olofsson, T.C. (2012) Symbions as major modulators of insect health: lactic acid bacteria and honeybees. PLoS One 7: e33188.

Wong, M.J., Liao, L.H., and Berenbaum, M.R. (2018) Biphasic concentration-dependent interaction between imidacloprid and dietary phytochemicals in honey bees (Apis mellifera). PLoS One 13: e0206625.

Yang, B., Qin, C., Hu, X., Xia, K., Lu, C., Gudda, F.O., et al. (2019) Enzymatic degradation of extracellular DNA exposed to chlorpyrifos and chlorpyrifos-methyl in an aqueous system. Environ Int 132: 105087.

Zhu, L., Qi, S., Xue, X., Niu, X., and Wu, L. (2020) Nitenpyram disturbs gut microbiota and influences metabolic homeostasis and immunity in honey bee (Apis mellifera L.). Environ Pollut 258: 113671.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M., and Van’t Riet, K.J.A.E.M. (1990) Modeling of the bacterial growth curve. Appl Environ Microbiol 56: 1875–1881.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.
Fig. S1 Pseudo-heat map showing the growth parameters ($A$ (Ab$_{620}$ units), $\mu_{\max}$ (Ab$_{620}$ units h$^{-1}$) and $\lambda$ (h)), carbon source utilization (fructose and sucrose) and exopolysaccharides (EPS) production by Fructobacillus fructose PL22, PL10 and PL25 and Apilactobacillus kunkeei PL13, BEE4 and BV61, which were incubated for 24 h at 30°C in synthetic media. In x axis, “_F” and “_S” suffix represent the fructose and sucrose synthetic medium, respectively. Rows are clustered using Euclidean distance and McQuitty linkage (red line). The color scale shows the differences between the standardized data.

Fig. S2 Growth parameters* of Apilactobacillus kunkeei BEE4, BV61 and PL13 under exposure to plant secondary metabolites (nicotine and p-coumaric acid) and agrochemicals (Mediator, Reldan and Roundup) for 24 h at 30°C. Data are the mean of three separate analyses ± standard deviations.

Table S1 Fructophilic lactobacilli strains used in this study.