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

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Summary

The alteration of a 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.

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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 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 (Gilliamella, 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 Ab_{620} units. Maximum growth rate (μ_{max}) and lag phase (λ) ranged from 0.07 $\pm$ 0.00 to 0.13 $\pm$ 0.01 Ab_{620} units h^{-1} and from 4.48 $\pm$ 0.12 to 9.66 $\pm$ 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 $\pm$ 0.01 Ab_{620} 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 $\pm$ 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*, μ_{max} and λ were in the range of 0.55–0.62 Ab_{620} units, 0.05–0.06 Ab_{620} 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 PL25 and *Apilactobacillus kunkeei* PL13, BEE4 and BV61, which were incubated for 24 h at 30°C.

	Fructose synthetic medium					Sucrose synthetic medium				
Samples	*A*	μ_{max}	λ	Fructose reduction (%)	EPS (g l^{-1})	*A*	μ_{max}	λ	Sucrose reduction (%)	EPS (g l^{-1})
Fructobacillus fructose PL10	0.43 ± 0.01^{d}	0.07 ± 0.00^{b}	4.93 ± 0.05^{c}	97.25 ± 0.23^{b}	0.17 ± 0.01^{ab}	–	–	–	–	–
F. fructose PL25	0.44 ± 0.01^{d}	0.08 ± 0.00^{b}	4.99 ± 0.07^{c}	97.89 ± 0.23^{b}	0.19 ± 0.01^{ab}	–	–	–	–	–
F. fructose PL22	0.51 ± 0.03 cd	0.08 ± 0.01^{b}	4.48 ± 0.12^{c}	96.73 ± 0.05^{b}	0.13 ± 0.01^{b}	–	–	–	–	–
Apilactobacillus kunkeei PL13	0.59 ± 0.01^{bc}	0.11 ± 0.00^{a}	8.61 ± 0.01^{b}	100.0 ± 0.00^{a}	0.19 ± 0.01^{ab}	0.55 ± 0.01^{b}	0.05 ± 0.00	6.20 ± 0.09^{b}	10.61 ± 2.08^{b}	0.35 ± 0.01^{b}
A. kunkeei BEE4	0.64 ± 0.00 ^{ab}	0.13 ± 0.00^{a}	9.66 ± 0.04^{a}	100.0 ± 0.00^{a}	0.23 ± 0.01^{a}	0.56 ± 0.00^{b}	0.06 ± 0.00	7.56 ± 0.08^{a}	21.83 ± 4.59^{ab}	0.33 ± 0.01^{b}
A. kunkeei BV61	0.71 ± 0.01^{a}	0.13 ± 0.01^{a}	8.32 ± 0.31^{b}	100.0 ± 0.00^{a}	0.16 ± 0.00^{ab}	0.62 ± 0.02^{a}	0.05 ± 0.00	7.52 ± 0.51^{a}	32.59 ± 4.79^{a}	0.43 ± 0.01^{a}

Data are the mean of three separate analyses ± standard deviations. Means within the column with different letters are significantly different ($P < 0.05$). –, not detected.
*. Growth kinetics was modelled according to the Gompertz equation as modified by Zwietering *et al.* (1990): $y = k + A \exp\{-\exp[(\mu_{max}e/A)(\lambda - t) + 1]\}$. When the turbidity ($Ab_{620}$) was the dependent variable to be modelled, *k* was the initial level of turbidity (Ab_{620} units), *A* was the difference in Ab_{620} units between inoculation and the stationary phase, μ_{max} was the maximum growth rate (Ab_{620} units h^{-1}), λ was the length of the lag phase (h), and *t* was the time (h).

large and dense mucus colonies on both FYP and mFYP agar media. Whereas less dense colonies were observed only on FYP agar media inoculated with *F. fructosus* strains (data not shown). The quantity of EPS produced in presence of fructose as carbon source ranged between 0.13 ± 0.01 and 0.19 ± 0.01 g l^{-1} for *F. fructose* strains, and between 0.16 ± 0.00 and 0.23 ± 0.01 g l^{-1} for *A. kunkeei* strains. Only *A. kunkeei* strains were able to produce EPS in presence of sucrose as carbon source (values between 0.33 ± 0.01 and 0.43 ± 0.01 g l^{-1}) (Table 1). Clustering analysis based on kinetics of growth, carbon source utilization and EPS production grouped the strains into 2 separate clusters (Fig. S1). *F. fructosus* strains were grouped in cluster A, whereas *A. kunkeei* strains in cluster B. Considering the performance and outcome of all the investigated parameters, three *A. kunkeei* strains were selected for further experiments.

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

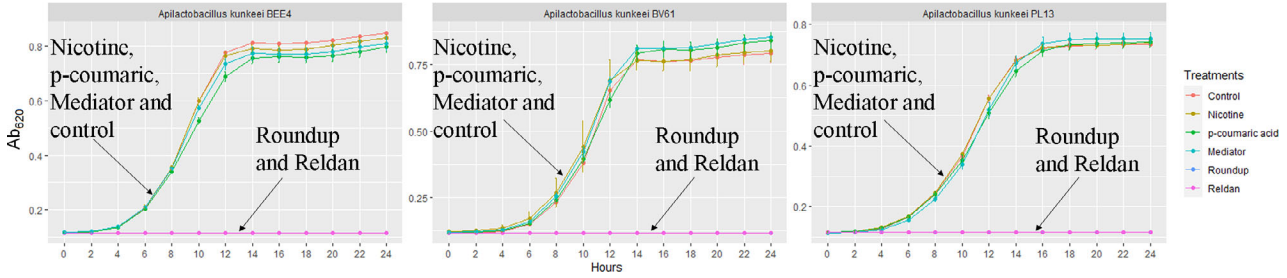


Fig. 1. Growth kinetics (Ab_{620}) of *Aplilactobacillus kunkeei* BEE4, BV61 and PL13 under exposure to plant secondary metabolites (nicotine and *p*-coumaric acid) and agrochemicals (Mediator, Reldan and Roundup) for 24 h at 30°C. Data are the mean of three separate analyses ± standard deviations.

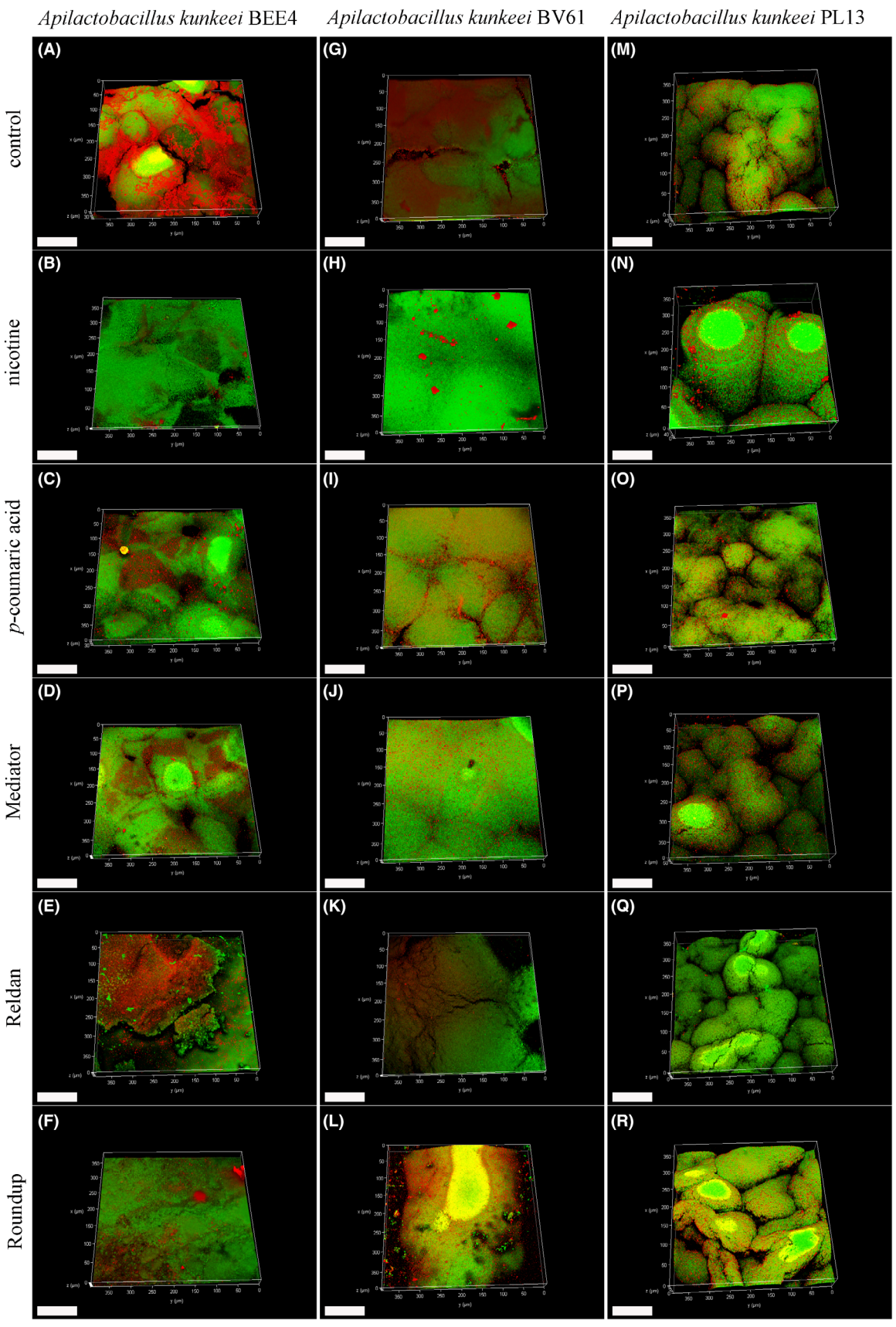
and PL13 respectively. When treated with Roundup, the cell density was 7.32 ± 0.15, 7.11 ± 0.15 and 7.14 ± 0.25 Log CFU g^{-1} for *A. kunkeei* BEE4, BV61 and PL13 respectively. The exposure to Mediator caused significant ($P < 0.05$) reduction of Log CFU g^{-1} only for *A. kunkeei* BEE4 (from 8.25 ± 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*

Fig. 2. Confocal microscopy analysis of biofilms by *Apilactobacillus kunkeei* BEE4 (panels A–F), BV61 (G–L) and PL13 (M–R) that were exposed, after grown, 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 exopolysaccharides in biofilm extracellular polymeric matrix show red fluorescence. Scale bars represent 100 μm; units of *x*, *y* and *z* axes are μm.



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 Reldan, they were 0.18 ± 0.06, 0.28 ± 0.04, 0.08 ± 0.05 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

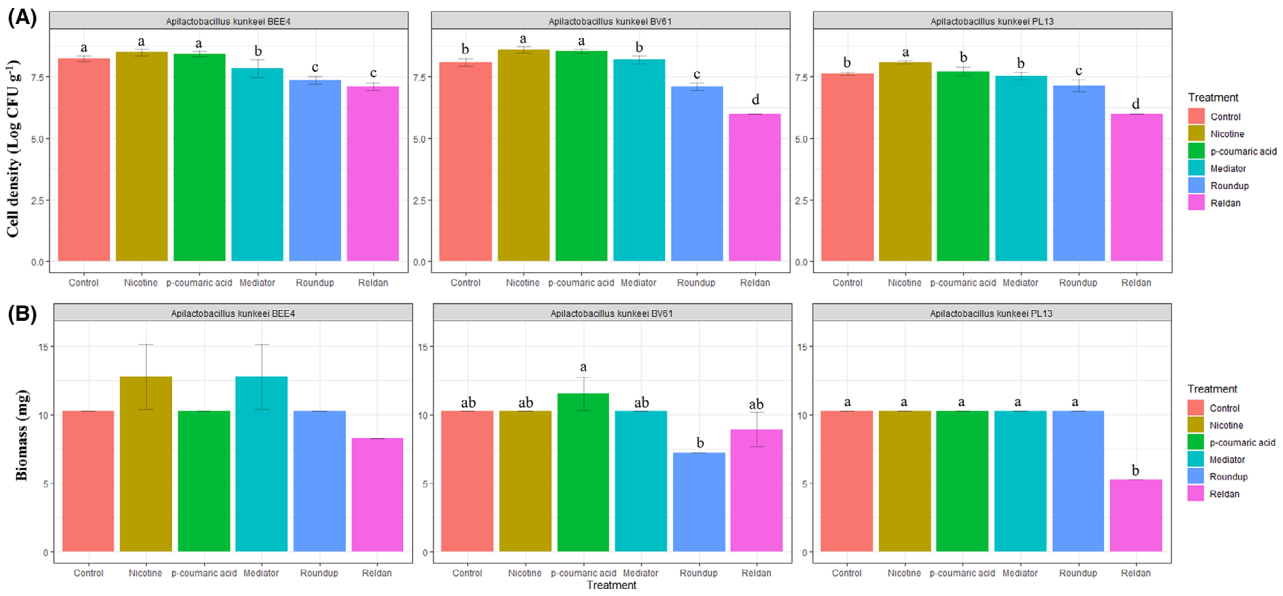


Fig. 3. Cell density (Log CFU g^{-1}) (A) and biomass (mg) (B) of *Apilactobacillus kunkeei* BEE4, BV61 and PL13 biofilms that, after 96 h of growth, were exposed to various plant secondary metabolites (nicotine and *p*-coumaric acid) and agrochemicals (Mediator, Reldan and Roundup). 'Control' indicates 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 ($P < 0.05$).

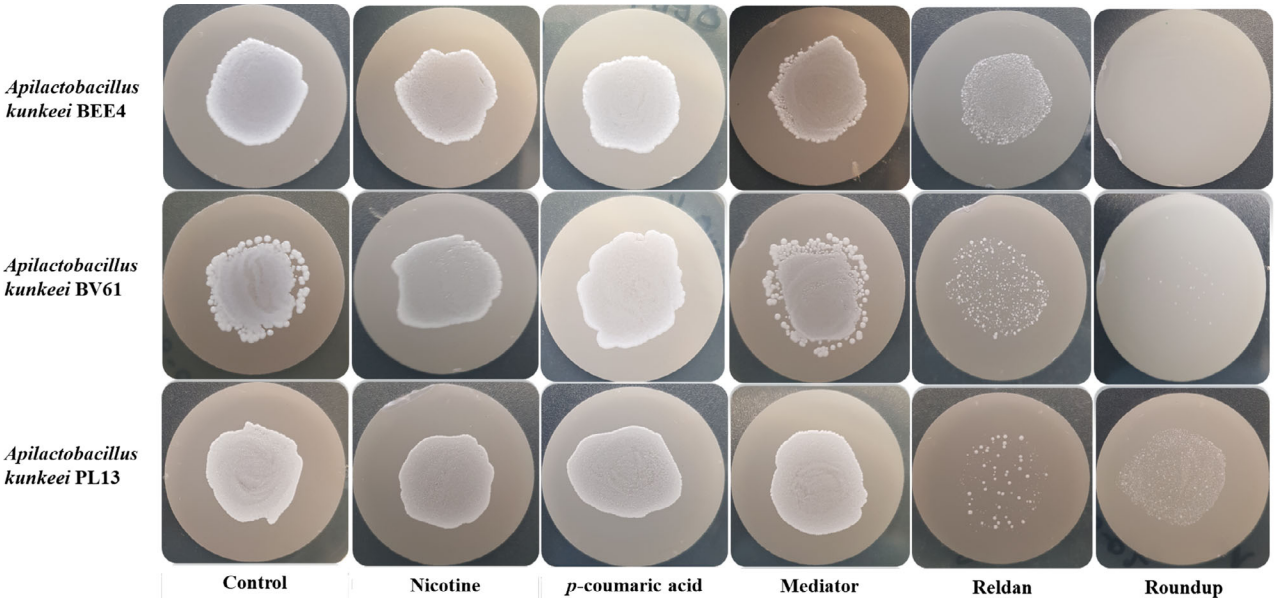


Fig. 4. *Apilactobacillus kunkeei* BEE4, BV61 and PL13 biofilms after 96 h of growth under exposure to various plant secondary metabolites (nicotine and *p*-coumaric acid) and agrochemicals (Mediator, Reldan and Roundup). Control indicates the control sample without supplementation of phytosanitary products and plant secondary metabolites.

were detected in *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.*, 2019; Liu *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 *F. fructosus* and *A. kunkeei* species and previously isolated from honeybee (*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 *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 *A. kunkeei* planktonic cells was completely inhibited by Roundup and Reldan, whereas nicotine, *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 *Enterococcus faecalis*, *Enterococcus faecium*, *Bacillus badius*, *Bifidobacterium adolescentis* and *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.*

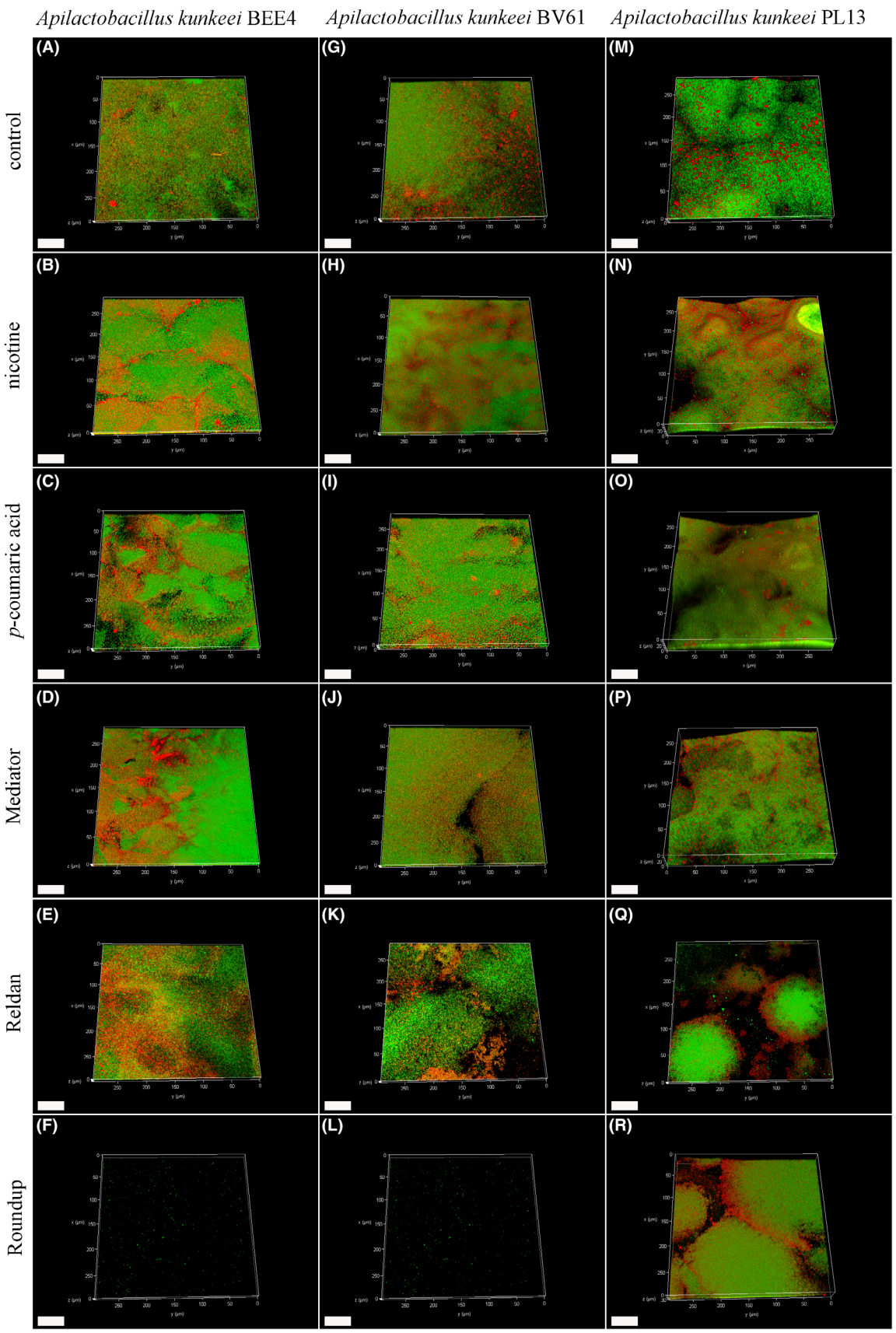


Fig. 5. Confocal microscopy analysis of biofilms produced by *Apilactobacillus kunkeei* BEE4 (panels A–F), BV61 (G–L) and PL13 (M–R) under exposure to nicotine (panels B, H, N), *p*-coumaric acid (C, I, O), Mediator (D, J, P), Reldan (E, K, Q) and Roundup (F, L, R). Panels A, G, M represent control biofilms 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.

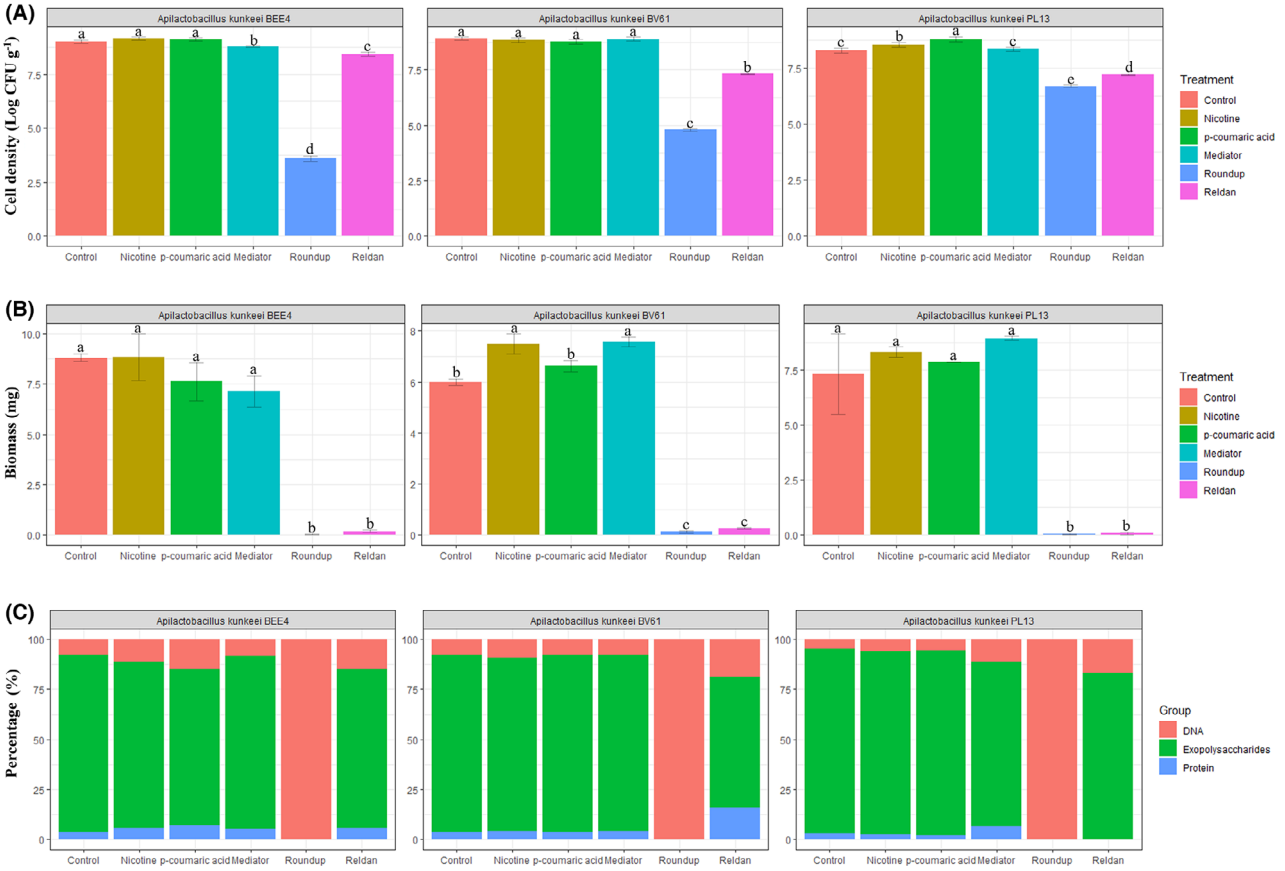


Fig. 6. Cell density (Log CFU g^{-1}) (A), biomass (mg) (B) and relative abundance of different extracellular polymeric matrix components (C) of *Apilactobacillus kunkeei* BEE4, BV61 and PL13 biofilms after 96 h of growth under exposure to various plant secondary metabolites (nicotine and *p*-coumaric 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 ($P < 0.05$).

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

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 (Slížová *et al*., 2015; Berríos *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 (Geldert *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 then 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 Polypeptone (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 (Ab_{620}). Kinetics of growth was determined and modelled according to the Gompertz equation as modified by Zwietering *et al.* (1990): $y = k + A \exp\{-\exp[(\mu_{max}e/A)(\lambda - t) + 1]\}$. When the Ab_{620} was the dependent variable to be modelled, k was the initial level of Ab_{620} units, A was the difference in Ab_{620} units between inoculation and the stationary phase, μ_{max} was the maximum growth rate (Ab_{620} units h^{-1}), λ 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 millilitres 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. *p*-Coumaric acid

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

	Active ingredient	Form	Concentration	References
Plant secondary metabolites	Nicotine	Pure compound	30 μM	Köhler *et al.* (2012)
	p-Coumaric acid	Pure compound	0.5 mM	Wong *et al.* (2018)
Agrochemicals	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 are 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. Ab_{620} 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 (Practum 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 vertex (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, Buccinasco, 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

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, Segrate, Italy) according to manufacturer's protocol. The measure was at 590 nm with Infinite F200 Pro (Tecan, Männedorf, Switzerland). Bovine serum albumin (BSA) was used as a standard. Biofilm EPS were quantified by phenol sulphuric acid method (Chiba *et al*., 2015). Briefly, 20 μl of ECM was mixed with 20 μl of 5% phenol in the 96 wells plate. Then, 100 μl of sulphuric acid was added and incubated for 10 min at 25°C. The concentration was measured at 492 nm with Infinite F200 Pro (Tecan). Glucose was used as a standard. All measures were performed in triplicate.

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.

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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.

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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), μ_{max} (Ab_{620} units h^{-1}) and λ (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 *Aplilactobacillus 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.

