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Impact of microbial communities on floral nectar chemistry: Potential implications for biological control of pest insects

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Abstract

Nectar-producing plants are increasingly used in agroecosystems to provide biological control agents (BCAs) such as predatory insects and parasitoids with necessary sugars to fulfil their nutritional requirements. However, it has recently been shown that nectar is commonly infested by microorganisms which may change the chemistry of the nectar and therefore its attractiveness and nutritional suitability for insects. The aim of this study was to investigate the impact of nectar-inhabiting microorganisms on nectar chemistry of three co-flowering plant species that are commonly used to provide BCAs with sugar resources (*Borago officinalis* L., *Centaurea cyanus* L. and *Symphytum officinale* L.). First, we assessed the abundance and visitation rate of flower-visiting insects on these plant species. Further, we identified the culturable microbes inhabiting the floral nectar and assessed changes in pH, total sugar concentration, main nectar sugars and amino acids. Flowers of all three plant species were found to be visited by a wide range of insects, with *B. officinalis* being the most attractive plant species for BCAs. Nectar microbial community structure differed significantly between plant species, irrespective of experimental field site. Microbial contamination affected both sugar and amino acid concentration and composition. Especially, sucrose and the fructan sugars 1-kestose and neokestose were found at significantly lower concentrations in contaminated nectar. Further, microbial contamination resulted in a significant increase of alanine and glycine, and a decrease in threonine and valine. Further research is needed to investigate the precise impact of these changes on the efficacy of biological control agents to control pest insects.

Zusammenfassung

Nektar-produzierende Pflanzen werden zunehmend in Agrarökosystemen eingesetzt, um Nützlinge (räuberische Insekten und Parasitoide) mit den notwendigen Zuckern zu versorgen. Indessen wurde vor kurzem gezeigt, dass Nektar gewöhnlich von Mikroorganismen besiedelt ist, die die Chemie des Nektars und damit seine Attraktivität und Eignung als Nahrung für Insekten verändern könnten. Das Ziel dieser Untersuchung war es, den Einfluss von Nektar-bewohnenden Mikroorganismen

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auf die Chemie des Nektars bei *Borago officinalis* L., *Centaurea cyanus* L. und *Symphytum officinale* L. zu untersuchen. Diese Pflanzenarten werden gemeinhin zur Unterstützung von Nützlingen eingesetzt. Wir bestimmten die Abundanz und Besuchsrate von blütenbesuchenden Insekten, identifizierten die kultivierbaren Mikroben aus dem Blütennektar und bestimmten Änderungen von pH, Gesamtzuckerkonzentration und der Konzentrationen der wichtigsten Nektarzucker und–aminosäuren. Blüten aller drei Arten wurden von einem breiten Spektrum von Insekten besucht, wobei *B. officinalis* für Nützlinge am attraktivsten war. Die Gemeinschaftsstruktur der Nektarmikroben war zwischen den Pflanzenarten signifikant unterschiedlich, wobei es keinen Effekt der Untersuchungsfläche gab. Mikrobielle Kontamination beeinflusste Konzentration und Zusammensetzung sowohl der Zucker als auch der Aminosäuren. Insbesondere Saccharose und die Fructanzucker 1-Kestose und Neokestose nahmen in kontaminiertem Nektar ab. Desweiteren bewirkte mikrobielle Kontamination eine signifikante Zunahme von Alanin und Glycin sowie eine Abnahme von Threonin und Valin. Weitere Untersuchungen werden benötigt, um die genaue Wirkung dieser Veränderungen auf die Effektivität der Nützlinge bei der Schädlingskontrolle zu ermitteln.

Keywords: Biological control agent; Biological pest control; Floral nectar

Introduction

Biological control programs that utilise biological control agents (BCAs; natural enemies), such as predatory insects and mites or parasitoids, to reduce the number of pest insects have become increasingly important in insect pest management. Predators, such as lady beetles and lacewings, are mainly free-living species that consume a large number of preys during their lifetime, thereby reducing the number of prey insects. Parasitoids, on the other hand, are species whose immature stage develops on or within a single insect host, ultimately killing the host. By far, the order of Hymenoptera (harbouring many parasitoid wasp species) comprises the largest group of parasitoids, and constitutes the overall most important group of natural enemies used in the biological control of insects. However, success of biological control largely depends on the availability of sugar-rich food sources, e.g. to cover the energetic needs of adult parasitoids or to supplement the diet of predators, especially in periods when insufficient preys are available (Wäckers & van Rijn, 2012). Therefore, supplying sugar resources such as specific sugar solutions (Jacob & Evans, 1998) or nectar-producing plants is more and more used to enhance BCA efficacy, both for naturally occurring BCAs and BCAs that are released in the environment to boost the presence of natural enemies (Gurr, Wratten, Tylianakis, Kean, & Keller, 2005; Winkler, Wäckers, Bukovinszkine-Kiss, & Van Lenteren, 2006). In contrast to pure sugar solutions, nectar has the advantage that it provides BCAs with essential amino acids as well, which can contribute to egg production and maturation (Chambers, 1988).

Floral nectar mainly consists of mono- and disaccharides of which sucrose, glucose and fructose are by far the most common and abundant. Additionally, other carbohydrates such as melezitose, maltose, raffinose and melibiose have been found in several nectars, although in low concentrations (Baker & Baker, 1983). It may be expected that nectar also contains (traces of) sugars that occur abundantly in other plant substances or tissues. One such group of plant sugars are the fructans, fructose-based oligo- and polymers, which function as reserve carbohydrates in many plant species (Hendry, 1993; Van Laere & Van den Ende, 2002), but their potential occurrence in nectar remains undescribed. Although sugars clearly dominate the total solutes in nectars, they also contain amino acids and other compounds such as lipids, minerals and vitamins, though at a much lower extent (Nicolson & Thornburg, 2007). While sugars represent a major energy source, amino acids can affect the attractiveness of nectar by providing taste to nectar (Gardener & Gillman, 2002; Nepi, 2014).

Although it has generally been assumed that the properties of nectar represent intrinsic plant features that are stable in time, recently it has been shown that nectar is commonly inhabited by microorganisms (see Pozo, Lievens, & Jacquemyn, 2015 for a recent overview) that may change the chemistry of nectar. For example, nectar-inhabiting microorganisms have been shown to alter the nectar sugar and/or amino acid composition and concentration and pH (Herrera, García, & Pérez, 2008; Canto & Herrera, 2012; Vannette, Gauthier, & Fukami, 2013), and therefore positively or negatively affect nectar attractiveness and usefulness for pollinators (Herrera, Pozo, & Medrano, 2013; Vannette, Gauthier & Fukami, 2013; Good, Gauthier, Vannette, & Fukami, 2014; Schaeffer, Phillips, Duryea, Andicoechea, & Irwin, 2014). Additionally, nectar-inhabiting microorganisms may contribute to overall flower scent (Golonka, Obi Johnson, Freeman, & Hinson, 2014) and contribute to insect attraction by odour production (Davis & Landolt, 2013). Altogether, these studies show that nectar-inhabiting microorganisms may have a major impact on the quality of nectar as a resource for pollinators. Likewise, it is reasonable to assume that nectar-inhabiting microorganisms may also affect the suitability of nectar for BCAs by changing the nectar chemistry, and therefore also the suitability of nectar-producing plants in biological control programs. The objective of this study was to investigate the impact of nectar-inhabiting microorganisms on nectar chemistry of three co-flowering plant species (*Borago officinalis* L.,

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Centaurea cyanus L. and *Symphytum officinale* L.) that are commonly included in commercial seed mixtures as nectar resources for BCAs. First, we assessed abundance and visitation rates of BCAs on these plant species grown in experimental plant communities. Secondly, we investigated whether the nectar of these plant species may be prone to microbial contamination and compared microbial community structure between species. Further, we investigated the impact of the nectar-inhabiting microorganisms on nectar chemistry by assessing changes in nectar sugars and amino acids. Finally, results are discussed in relation to the potential impact on BCA efficacy.

Material and methods

Studied plant species and experimental plant communities

Three nectar-producing plant species that are commonly used in commercially available seed mixtures for establishing nectar-rich field margins were investigated in this study: *B. officinalis* L. ('starflower' or 'borage'; Boraginaceae), *C. cyanus* L. ('cornflower'; Asteraceae) and *S. officinale* L. ('common comfrey'; Boraginaceae). In spring 2013 (week 21), each plant species was sown (5 g/m^2) in artificial flower strips (4.50 × 1 m; one strip per species), separated by a 1-m-wide non-nectar producing grass strip. Plots were replicated at two experimental sites that were roughly two kilometres away from each other. The study sites were located in an agricultural environment in Pulderbos (Site 1: N51°13′18.2″, E4°41′34.4″, 6.0 m a.s.l.; Site 2: N51°13′27.4″, E4°42′42.3″, 9.0 m a.s.l.; Antwerp, Belgium): site 1 was partly surrounded by maize and partly by grassland; site 2 was completely surrounded by open grass fields. As far as we know, no managed pollinators or commercial BCAs were introduced at either site. When plants started flowering, one part of the flowers was left un-manipulated, keeping the nectar accessible for visiting insects (treatment "unbagged"), while in a second treatment flowers were excluded from visiting insects (treatment "bagged") by which microbial nectar contamination is generally avoided (Pozo, Lachance, & Herrera, 2012). Inflorescences were bagged with a fine mesh from the bud stage on (mesh size 0.27 mm × 0.88 mm). During the entire study, no agrochemicals were applied.

Monitoring flower-visiting insects

To determine the activity and identity of flower-visiting insects, censuses of flower visitors in our study plots were carried out as described previously (Herrera, Sánchez-Lafuente, Medrano, Guitián & Rey, 2001). Censuses (observation period of 3 min) were performed weekly by trained observers between 8:00 h and 21:00 h on sunny, dry days from late July until early September and the abundance and visitation rate of flower-visiting insects (flower visits per minute) were determined. Five categories of flower visitors were distinguished: (i) BCAs (defined here as parasitoid wasps (Hymenoptera) and hoverflies (Syrphidae; Diptera) whose larvae can be important predators of pest insects, (ii) social bees (Hymenoptera), (iii) butterflies (Lepidoptera), (iv) flies (Diptera) and (v) other insects. Both parasitoid wasps (including species of *Cotesia*, *Aphidius*, *Microplitis* and many others) and hoverflies strongly depend as adults on sugar-rich food sources such as nectar to fulfil their nutritional requirements (Jervis, Kidd, Fitton, Huddleston, & Dawah, 1993). In total, 43 and 67 censuses were conducted for experimental site 1 and 2, respectively.

Nectar sampling

During peak flowering, for each plant species nectar samples were collected from both study sites over a period of two or three consecutive weeks (see Appendix A: Table S1). In total, for each plant species, ten individuals were sampled from plants exposed to insect visitation. In addition, five individuals were sampled from the "bagged" treatment. More particularly, for each plant individual a total of 10 μL floral nectar was collected by combining nectar from multiple flowers of the same age (mean recorded values of 0.9 μL, 2.6 μL and 3.5 μL per flower for *C. cyanus*, *B. officinalis* and *S. officinale*, respectively). Sampled flowers were less than one day old for *B. officinalis* and less than three days old for *C. cyanus* and *S. officinale*. Following measurement of the sugar concentration (expressed in degrees Brix) using a hand refractometer, nectar samples were subjected to microbial and physicochemical analysis. To avoid contamination between measurements, the refractometer was cleaned with 70% ethanol.

Microbial analysis

Within 24 h after nectar sampling, 1/30 diluted (in sterile milli-*Q* water) nectar samples (10 samples per species for the "bagged" treatment, five samples per species for the "unbagged" treatment) were plated on two media (50 μL per plate): (i) trypticase soy agar (TSA; Oxoid, Basingstoke, UK) and (ii) yeast extract glucose agar (YGC; Sigma-Aldrich, Steinheim, Germany) supplemented with 0.5 g/L chloramphenicol. Although not specifically optimised for culturing microbes from nectar, these media represent a general growth medium for bacteria and a specific medium for yeasts, respectively, and have been successfully used previously for isolating diverse microorganisms from nectar (e.g. Álvarez-Pérez, Herrera, & de Vega, 2012; Pozo, Lachance & Herrera, 2012; Jacquemyn et al., 2013a; Jacquemyn, Lenaerts, Tyteca, & Lievens, 2013b). After plating, plates were incubated at 25 °C for 7 days. Subsequently, from each plate, one colony was picked for each morphologically distinct colony type, and further subcultivated to obtain pure cultures. Bacterial and yeast isolates were preserved at −80 °C in trypticase

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soy broth and yeast extract peptone dextrose broth containing 37.5% glycerol, respectively. Identifications were performed by partially sequencing the small subunit ribosomal RNA (rRNA) gene (bacteria) and the D1/D2 domain of the large subunit rRNA gene (yeasts) as described previously (Jacquemyn et al., 2013a,; Jacquemyn, Lenaerts, Tyteca & Lievens, 2013b). Obtained sequences were compared with the nt database in GenBank (excluding uncultured bacteria, unclassified sequences and environmental samples), and isolates were assigned to the highest taxonomic rank possible. Further, sequences were grouped in operational taxonomic units (OTUs) using Mothur v.1.33.3 (Schloss et al., 2009) based on a dissimilarity cut-off value of 1%, allowing us to perform further analyses with species-level OTUs (Álvarez-Pérez, de Vega, & Herrera, 2013; Jacquemyn, Lenaerts, Tyteca & Lievens, 2013b). Representative sequences (as determined by Mothur) for each OTU were deposited in GenBank under the accession numbers KP405839–KP405918.

pH measurement and analysis of nectar sugar and amino acids composition

The pH of all nectar samples was determined with pH-indicator paper (MERCK, Darmstadt, Germany). Additionally, a number of nectar sugars and amino acids were determined by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Thermo Fisher Scientific Dionex, Sunnyvale, CA). In addition to the three main nectar sugars (fructose, glucose and sucrose), a number of fructans known to occur in Asteraceae and Boraginacea (e.g. 1-kestose, 6-kestose, bifurcose and neokestose) (Van Laere & Van den Ende, 2002) were measured. Nectar samples were diluted with 10 μL sodium azide–water (0.02% w/v) and analysed as outlined earlier (Gijbels, Van den Ende, & Honnay, 2014). For each plant species, five samples were analysed for each treatment ("bagged" and "unbagged").

Data analysis

Non-parametric Kruskal–Wallis tests were used to see whether visitation rates of flower-visiting insects differed between (i) insect groups, (ii) plant species, (iii) experimental sites and (iv) time of census. In order to assess the overall richness of OTUs in the studied plant species, rarefaction curves were generated for each plant species using the vegan package. The expected OTU richness for each plant species was estimated by Chao1. To visualise differences in nectar microbial community structure between the three studied plant species as well as between study sites, non-metric multidimensional scaling (NMDS) was applied using Bray-Curtis distances (presence/absence of the identified OTUs). To test whether microbial communities in floral nectar differed between plant species or sites, an ANOSIM test was run with 9999 permutations. To test whether plant species, experimental site and treatment ("bagged" versus "unbagged") and the interactions between plant species and treatment, and between plant species and experimental site affected nectar physicochemistry (sugar and amino acids composition, pH and sugar concentration (Brix)), univariate analysis of variance (ANOVA) was used. Except for the Brix data, which were normally distributed, we took the physicochemical data plus 1 as dependent variable, with lognormal as distribution and identity as link. Differences were considered significant if $p \leq 0.05$. To visualise differences in nectar traits, a principal component analysis (PCA) was performed using all measured nectar traits and scores of the two first principal components were plotted against each other. Additionally, dot plots were constructed to visualise differences in the concentrations of individual sugars and amino acids for both treatments (SigmaPlot version 12.3). A ternary diagram for the main nectar sugars, i.e. fructose, glucose and sucrose, was constructed. All statistical analyses were conducted using the software program R.

Results

Monitoring insects in the field

Flowers of all three investigated plant species were visited by a wide range of insects belonging to the five categories defined. Observed BCAs were represented by species of Syrphidae and parasitoids such as *Cotesia glomerata*. Further, several pollinators (e.g. *Apis mellifera*, *Bombus hypnorum*, *B. lapidarius*, *B. lucorum*, *B. terrestris*, species of *Psithyrus* and butterflies), flies and other insects were observed. Insect abundance and visitation rate were significantly affected by plant species for the BCA category (Table 1; no significant difference between sites), with *B. officinalis* being the most attractive plant species for BCAs (i.e. most recorded BCA visits). Syrphidae visited all three investigated plant species, while only *C. cyanus* and *S. officinale* flowers were visited by parasitoids such as *Cotesia glomerata.* There were no significant differences between plant species and experimental sites for the other investigated categories (butterflies, flies and other insects), except for social bees (Table 1).

Nectar-inhabiting microorganisms

No microbes were found in nectar samples from bagged flowers used in this study (further referred to as "pristine" nectar). Regarding samples from flowers open to insect visitation, bacteria were found in all samples investigated, whereas yeasts were found in eight, nine and ten investigated samples for *S. officinale*, *B. officinalis* and *C. cyanus*, respectively. Colony counts on plates showed that microbial growth ranged from 10^2 to $>10^3$ (uncountable plate) colony-forming units (cfu) per μL nectar for bacteria and from 10^1 to 10^2 cfu/μL for yeasts, with highest microbial densities observed in nectar from *C. cyanus*. Direct cell counts from nectar under the

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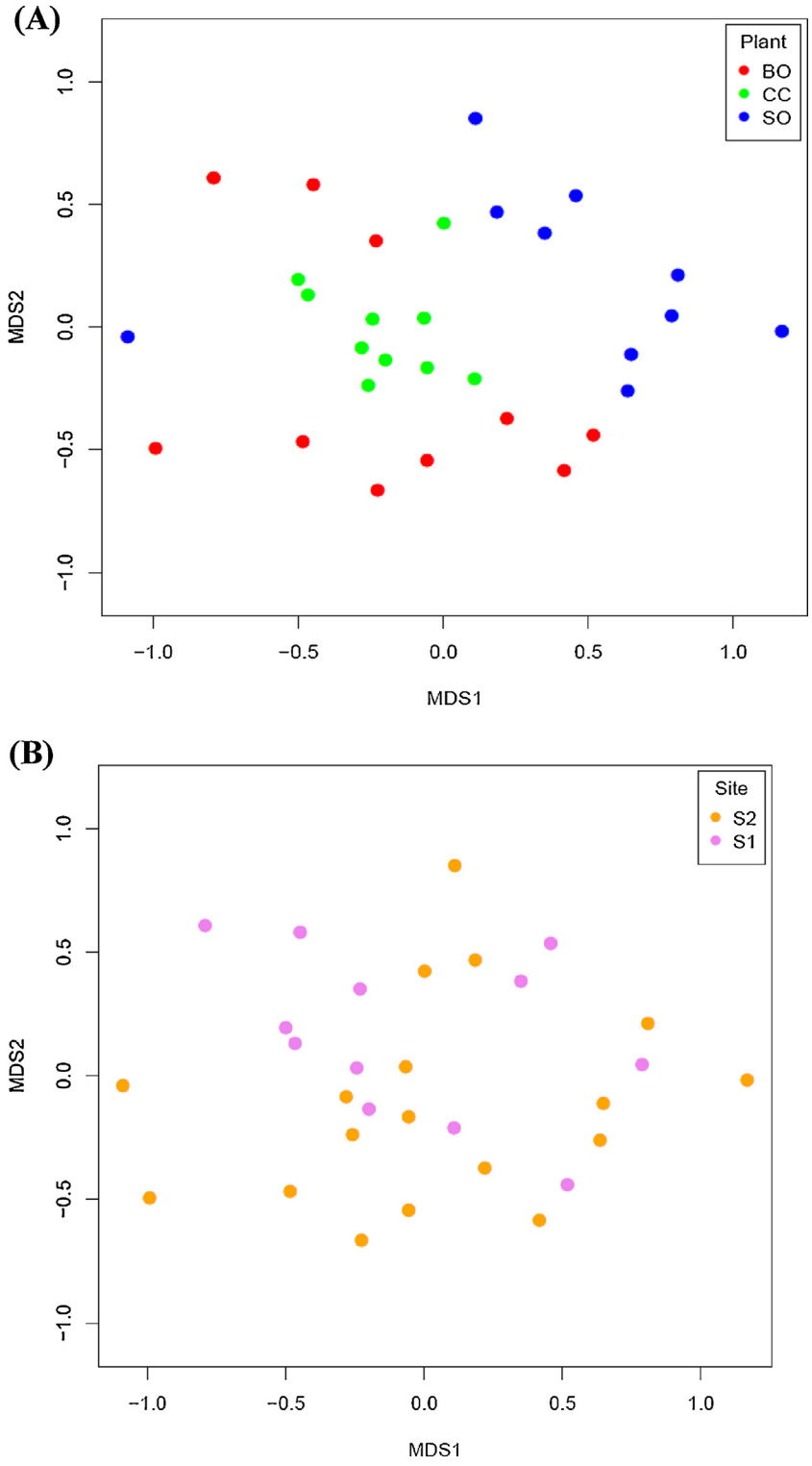

Fig. 1. NMDS ordination of the bacterial and yeast communities (presence/absence data) in floral nectar of *Borago officinalis* (BO), *Centaurea cyanus* (CC) and *Symphytum officinale* (SO) (A), obtained from two different study sites (Site 1 (S1) and Site 2 (S2)) (B). Microbial communities were characterised using species-level operational taxonomic units (OTUs), defined using a DNA dissimilarity cut-off value of 1%. Plant species was found as a significant factor (Anosim statistic $R = 0.35$; $p = 0.00010$); study site was not a significant factor (Anosim statistic $R = 0.010$; $p = 0.39$).

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Table 1. Summary of significance tests for the effects of plant species (including *Borago officinalis*, *Centaurea cyanus* and *Symphytum officinale*) and experimental sites (Site 1 and Site 2) on the activity of biological control agents (BCA; including species of Syrphidae and parasitoids, such as *Cotesia glomerata*), social bees (i.e. *Apis mellifera*, *Bombus hypnorum*, *B. lapidarius*, *B. lucorum*, *B. terrestris*, species of *Psithyrus*), butterflies, flies and other flower-visiting insects, data from 3-min censuses carried out during summer 2013.

		Significance test[a]		
Dependent variable	Classification variable	df	χ^2	p
Activity BCA	Plant species	2	5.88	**0.050**
	Experimental site	1	1.48	0.220
Activity social bees	Plant species	2	11.19	**0.004**
	Experimental site	1	9.63	**0.002**
Activity butterflies	Plant species	2	3.69	0.160
	Experimental site	1	0.030	0.860
Activity flies	Plant species	2	0.17	0.920
	Experimental site	1	0.91	0.340
Activity other	Plant species	2	2.81	0.250
	Experimental site	1	1.22	0.270

[a] Kruskal–Wallis test for estimating significant differences in median activities.

microscope were not made, but could have given a more accurate view on the bacterial and yeast densities (Brysch-Herzberg, 2004). In total, 58 bacterial OTUs were identified (see Appendix A: Table S2). Bacteria belonged to four phyla, including Proteobacteria (42 OTUs), Firmicutes (10 OTUs), Actinobacteria (5 OTUs) and Bacteroidetes (1 OTU). In total, 25, 29 and 35 bacterial OTUs were observed for *C. cyanus*, *B. officinalis* and *S. officinale*, respectively, representing 54.7 (*B. officinalis*), 56.2 (*C. cyanus*) and 67.7% (*S. officinale*) of the estimated OTU richness in the nectar (based on Chao1 richness estimations; Fig. S1B). Further, 22 yeast OTUs were identified, comprising both ascomycetous and basidiomycetous yeasts (see Appendix A: Table S3). In total, 12 yeast OTUs were found for *B. officinalis* and *C. cyanus*, 7 OTUs were found for *S. officinale*, representing 58.3 (*S. officinale*), 70.6 (*C. cyanus*) and 93.0% (*B. officinalis*) of the estimated yeast richness (Fig. S1B). NMDS analysis for the different plant species and experimental sites and subsequent ANOSIM analyses indicated significant differences in nectar microbial community composition between plant species, but not between experimental sites (Fig. 1A) ($R = 0.35$; $p = 0.00010$ and $R = 0.010$; $p = 0.39$, respectively), indicating that the studied plant species harboured a different microbial community, irrespective of the location (Fig. 1B).

Physicochemical nectar properties

Nectar pH (range 6.1–8.0) was significantly different between plant species (see Appendix A: Table S4) with the highest pH (pH = 8.0) in *B. officinalis* nectar. Sugar concentration (expressed as degrees Brix) was significantly affected by plant species, treatment and their interaction (see Appendix A: Table S4). Mean (±SE) Brix values in pristine nectar were 41.02 ± 1.58, 25.84 ± 3.06 and 23.74 ± 1.42, and 21.74 ± 2.67, 4.32 ± 0.37 and 13.92 ± 0.37 °Bx in contaminated nectar for *S. officinale*, *C. cyanus* and *B. officinalis*, respectively (see Appendix A: Table S5). HPAEC-PAD analysis showed that the concentration of individual sugars was mainly affected by plant species (significant for 3 out of 7 investigated sugars (fructose, glucose and sucrose)) and treatment (significant for 1-kestose, neokestose and sucrose). The interaction between plant species and treatment was only significant for 6-kestose (see Appendix A: Table S4). The first two principal components of the PCA performed on the nectar sugars explained 69.2% of the total variance. Sucrose (strongly correlated to PCA1) was the most prevalent carbohydrate in pristine nectar; in nectar infested with microorganisms glucose and fructose were the most prevalent sugars (Fig. 2B, see Appendix A: Fig. S2A and Table S5). Additionally, compared to pristine nectar, contaminated nectar had significantly lower concentrations of the fructans 1-kestose and neokestose (see Appendix A: Fig. S2A and Table S5). Interestingly, HPAEC-PAD analysis also revealed an array of saccharides with retention times that did not correspond to any of the known fructans (see Appendix A: Fig. S3). Similar to fructans, these compounds were sensitive to mild acid hydrolysis, suggesting that they may represent hetero-oligosaccharides that contain some fructosyl–fructose linkages. Similarly, amino acid concentration was mainly affected by plant species (significant for 13 out of 20 investigated amino acids), by site (7 out of 20) and treatment (4 out of 20). Interactions were almost never significant (see Appendix A: Table S4). The first two principal components of the PCA performed on the amino acids explained 51.5% of the total variance. In general, the difference between contaminated and pristine nectar could be attributed to PCA2 (mainly correlated with alanine and glycine) (Fig. 3). The amino acids asparagine, threonine and valine dominated in pristine nectar. Threonine and valine concentrations

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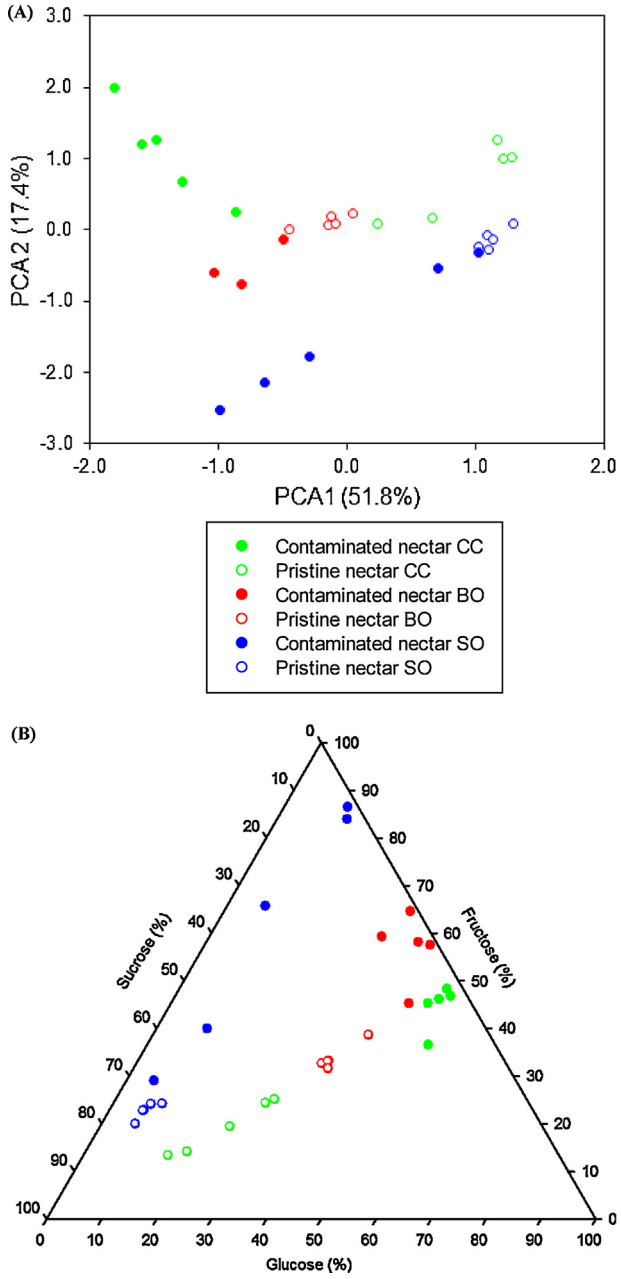

Fig. 2. Variation in sugar composition in pristine and contaminated nectar from *Borago officinalis* (BO), *Centaurea cyanus* (CC) and *Symphytum officinale* (SO). (A) PCA plot for the two first largest PCA components for all sugars analysed in this study (#7, see Table Table S5 for an overview). (B) Ternary diagram presenting the percentage of glucose, fructose and sucrose in the analysed samples. Open and shaded circles represent pristine and contaminated nectar, respectively. Data were obtained from five plants for each treatment.

were significantly lower in contaminated nectar (except for *C. cyanus*, where valine concentration increased). Further, significantly higher concentrations of alanine (except for *S. officinale* nectar in which alanine concentration decreased) and glycine were observed in contaminated nectar (see Appendix A: Fig. S2B, Table S5).

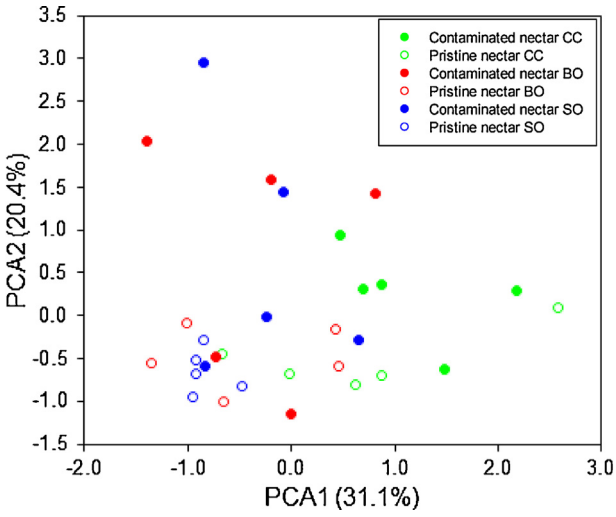

Fig. 3. Variation in amino acid composition in pristine and contaminated nectar from *Borago officinalis* (BO), *Centaurea cyanus* (CC) and *Symphytum officinale* (SO). PCA plot for the two first largest PCA components for all amino acids (#20, see Table S5 for an overview) analysed in this study. Open and shaded circles represent pristine and contaminated nectar, respectively. Data were obtained from five plants for each treatment.

Discussion

Attraction of BCAs

The plant species showing most BCA visits was *B. officinalis*, followed by *C. cyanus* and *S. officinale.* In our study, hoverflies (Diptera: Syrphidae) represented the most common BCAs visiting all three investigated plant species. Additionally, the parasitoid *Cotesia glomerata* was found on *S. officinale* and *C. cyanus.* These plant species have been shown to increase the longevity of several BCAs in laboratory experiments previously (Wäckers & van Rijn, 2012). Altogether, these features render them promising plant species to support BCAs in field conditions.

Nectar microbial communities

Whereas no microbes were found in the nectar of bagged control plants, nectar of flowers open to insect visitation harboured several bacterial and yeast OTUs. In these samples, bacteria were commonly present, whereas yeasts were less frequently observed. Additionally, yeasts were encountered at lower densities than bacteria, demonstrating that bacteria dominated our nectar samples. In line with previous research (Álvarez-Pérez, Herrera, & de Vega, 2012; Fridman, Izhaki, Gerchman, & Halpern, 2012; Jacquemyn et al., 2013a; Jacquemyn, Lenaerts, Tyteca, & Lievens, 2013b) the most prevalent bacterial families encountered encompassed the families Enterobacteriaceae, Bacillaceae and Pseudomonadaceae. These families not only represented

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the most widespread families among the sampled plant individuals and species, but also occurred at highest densities in the different nectar samples. Further, these families have been shown to be involved in interactions with insects (Leroy et al., 2011). The most prevalent yeasts encountered in this study included members of *Cryptococcus*. Several species within this genus commonly occur in flowers (Sandhu & Waraich, 1985), and may become inoculated into the nectar by, for example, contact with the corolla or visiting insects, despite not being truly specialised in nectar (Pozo, Lachance, & Herrera, 2012). Typical nectar yeasts such as *Metschnikowia* (Pozo, Lievens, & Jacquemyn, 2015) were also observed in nectar of *S. officinale*. It has to be noted that only culturable microorganisms were investigated in this study by which our view on the microbial communities in our nectar samples could be biased. A molecular study using a culture independent technique such as 454 pyrosequencing or Illumina MiSeq sequencing may give us a more accurate picture of the microbial communities occurring in nectar (Fridman, Izhaki, Gerchman, & Halpern, 2012).

Nectar-inhabiting microbes change physicochemical nectar properties

In agreement with previous studies (Herrera, García, & Pérez, 2008; de Vega, Herrera, & Johnson, 2009; Canto & Herrera, 2012; Vannette, Gauthier, & Fukami, 2013), microbial contamination reduced total sugar concentration. Especially sucrose concentration decreased significantly. Whereas fructose and glucose concentrations were similar in contaminated nectar of *C. cyanus*, *B. officinalis* and *S. officinale*, nectar contained more fructose than glucose. This may be explained by the abundant presence of bacteria in *S. officinale* nectar, resulting in fructose-dominated nectar (Vannette, Gauthier, & Fukami, 2013). Further, more variability was observed in the sugar composition of the nectar of the investigated *S. officinale* plants than in that of the other two species after infestation, maybe due to the higher variability in microbial communities in these samples. Similarly, the fructans 1-kestose and neokestose appeared in significantly lower concentrations in contaminated nectar, suggesting that these fructose polymers were utilised by microbes. Interestingly, although the plant-specific fructan profile of *Symphytum* (Van Laere & Van den Ende, 2002) was reflected in its nectar sugar profile, partial differential hydrolysis of fructans by microbial invertases likely occurred (Fig. S2A). Surprisingly, pH was not strongly affected by microbial contamination. Nevertheless, laboratory experiments have shown that microorganisms can have a substantial impact on the pH of nectar (Vannette, Gauthier, & Fukami, 2013), but this effect may be less pronounced at microbe densities as low as those observed in our study.

Similar to sugar concentrations, we found that the concentration of certain amino acids was significantly different between pristine and contaminated nectar, indicating that microbial contamination can have a broad impact on amino acids concentrations. For example, threonine and valine concentrations were lower in contaminated nectar, whereas alanine and glycine concentrations were higher. These results are in line with earlier laboratory experiments that have shown that nectar-dwelling yeast species are able to reduce the concentration of amino acids, with differences between species in the degree of consumption (Peay, Beslisle, & Fukami, 2012).

Potential implications of nectar-inhabiting microbes on BCA efficacy

We have shown that nectar microbes are able to alter nectar chemistry substantially, which in turn may affect BCA efficacy. It can therefore be expected that nectar-inhabiting microorganisms play an important role in the interactions between nectar-producing plants, BCAs and pest insects. However, it remains to be investigated whether these microbe-mediated effects are either beneficiary or detrimental to the BCAs. It has been shown before that activity of specific nectar microbes may increase the palatability of nectar for pollinators (Herrera, Pozo, & Medrano, 2013; Schaeffer, Philips, Duryea, Andicoechea, & Irwin, 2014). Nevertheless, it has also been shown that particular microbes may decrease nectar attractiveness for pollinators (Vannette, Gauthier, & Fukami, 2013; Good, Gauthier, Vannette, & Fukami, 2014). Given the fact that BCAs may have a strong preference for certain sugars, or only show a feeding response when a given concentration is reached (Makatiani, Le, Olson, Wäckers, & Takasu, 2014), it can be assumed that nectars depleted in certain sugar types or sugar concentration may be less efficient to support BCAs. However, further research is needed to draw strong conclusions in this regard. Further, since amino acids are an important nitrogen source for pollinators and BCAs (Nicolson & Thornburg, 2007), any change in amino acid content can be expected to have an impact on BCA activity. In general, insects require amino acids for growth, development, and egg production. Several parasitoid species can obtain these amino acids from their larval stage, but for optimum egg production, many species should ingest them as adults, through host feeding, nectar consumption or other resources (Panizzi & Parra, 2012). Our results revealed that nectar-inhabiting microorganisms have a significant impact on the concentration of at least two essential amino acids, including threonine and valine (both decreased), while alanine and glycine both increased. However, the exact role of the different microorganisms in affecting BCA behaviour and efficacy remains unclear so far. We therefore suggest that future studies should focus on (i) the feeding behaviour of BCAs, (ii) the impact of individual microbes on the feeding behaviour of the BCAs and (iii) the impact of microbial contamination on the behaviour of the pest insects. We are convinced that a better understanding of the ecological role of nectar-dwelling microorganisms will lead to enhanced biological control in agricultural cropping systems.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.baae.2015.10.001.

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