Nectar microbes may indirectly change fruit consumption by seed-dispersing birds

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Abstract

An increasing number of recent studies show that nectar-inhabiting microorganisms influence plant fitness by mediating interactions between plants and pollinators. However, whether the effects of nectar microbes extend beyond pollination to affect subsequent stages of plant reproduction remains largely unknown. This study aims to explore whether nectar microbes can indirectly affect fruit consumption by birds, which can be essential for seed dispersal and germination. Wild flowers of *Eurya japonica* trees were experimentally inoculated with the nectar-inhabiting yeast *Metschnikowia reukaufii* and the nectar-inhabiting bacterium *Acinetobacter boissieri*, both of which had been previously isolated frequently from *E. japonica* flowers. In this experiment, I examined whether these microbes changed female reproductive success of the understory tree. Experimental inoculation of flowers with yeasts decreased fruit and seed set compared to those inoculated with bacteria, and the control. Furthermore, fruits with higher seed set tended to be larger, and larger fruits were more likely to be consumed by seed-dispersing birds, including the Japanese white-eye *Zosterops japonica,* the brown-eared bulbul *Hypsipetes amaurotis*, and the Daurian redstart *Phoenicurus auroreus*. These results suggest that nectar-inhabiting microorganisms have the potential to affect plant reproduction by influencing not only plant-pollinator interactions, but also by indirectly modifying plant-frugivore interactions via changes in plant-pollinator interactions.

Introduction

An increasing number of studies over the past decade have shown that nectar-inhabiting microorganisms can mediate plant-pollinator interactions by modifying floral traits (Álvarez-Pérez et al., 2013; De Vega & Herrera, 2013; Vannette et al., 2013; Rering et al., 2017, 2020, 2021;

Rebolleda-Gómez et al., 2019; Schaeffer et al., 2019). For example, the dominant nectar yeast *Metschnikowia reukaufii* increases pollen donation in *Delphinium nuttallianum* (Schaeffer & Irwin, 2014) via enhanced pollinator attraction. In contrast, the nectar bacterium *Neokomagataea* sp. reduces pollinator attraction and seed production in *Diplacus auran-tiacus* (Vannette et al., 2013; Vannette & Fukami, 2018) via decreased nectar pH (Chappell et al., 2022). These studies suggest that microorganisms are players in pollination mutu-alisms that influence plants and pollinators on ecological and likely evolutionary timescales (Vannette, 2020).

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While microorganisms are known to alter pollination success, seed set, and seed mass (Herrera et al., 2013), their effect on plant-frugivore interactions remains unknown. Although seed and fruit set are often used as indicators of female reproductive success of plants (Vannette et al., 2013; Tsuji & Ohgushi, 2018), many plants cannot complete reproduction without frugivorous animals that disperse seeds (Krefting & Roe, 1949; Traveset, 1998; Traveset et al., 2001). Many plant species that depend on animals for seed dispersal can germinate only after seeds pass through a fruit-consuming animal (Herrera, 1989; Krefting & Roe, 1949; Traveset, 1998; Traveset et al., 2001; Ruxton & Schaefer, 2012).

If nectar microbes mediate flower-pollinator interaction and change plant fitness, they might also mediate fruit-frugivore interactions indirectly. In this study, I sought to understand potential effects of nectar microbes on fruit consumption by seed-dispersing animals. To that end, I did field experiments using *Eurya japonica* trees and microorganisms in its' floral nectar. This plant needs fruit consumption by birds to germinate (Manabe et al., 1992), and yeasts and bacteria inhabit nectar (Tsuji & Fukami, 2018), though it is unknown whether the microbes affect reproductive success of this plant. I first examined whether and how the nectar-inhabiting yeast *M. reukaufii* and the nectar-inhabiting bacterium *Acinetobacter boissieri* change fruit and seed set in the tree. I also quantified the extent to which seed set affected fruit size. Finally, I investigated whether fruit size influenced the likelihood of fruit consumption by seed-dispersing birds.

Materials and methods

Study plant

Eurya japonica (Pentaphylacaceae) is a common subdioecious tree native to much of Japan and other parts of East Asia (Chung & Chung, 2000; Tsuji & Sota, 2011; Wang et al., 2016). The understory trees inhabit forests dominated by *Pinus* and *Quercus* (Sugimura & Yata, 2003). Trees used in this study were located at the same wood edge site, where *E. japonica* occurred abundantly, in Kozagawa, Wakayama Prefecture, Japan (33° 31' 50" N, 135° 49' 00" E), as used by Tsuji and Sota (2010, 2013), Tsuji and Fukami (2018), and Tsuji and Ohgushi (2018).

At this site, this species blooms in early spring and is visited by a diversity of pollinators including flies, bees, wasps, and midges, with Diptera being the dominant visitors (Tsuji & Ohgushi, 2018; Tsuji et al., 2020), though relative efficiency of pollen transfer of each pollinator taxon is unknown. These insects carry microorganisms between flowers (Tsuji & Fukami, 2018). The number of ovules, which are potential seeds, varies widely among flowers (Tsuji & Ohgushi, 2018). In this study, I counted from 1 to 41 ovules per flower (on average 16 +/- 6 SD). After fertilization, fruits continue to develop and slowly increase in size until late fall. In winter, ripe fruits are consumed by seed-dispersing birds, including

the brown-eared bulbul (*Hypsipetes amaurotis*), the pale thrush (*Turdus pallidus*), the white-cheeked starling (*Sturmus cineraceus*), the Japanese white-eye (*Zosterops japonica*), and the Daurian redstart (*Phoenicurus auroreus*), among others (Manabe et al., 1992, Fig. 1A, Appendix A: Videos S1-S2, Table S1). It has been confirmed that seeds within pericarp have a low germination rate (Manabe et al., 1992). That is, fruits not eaten by birds do not contribute to seedling recruitment (Manabe et al., 1992).

Microbial inoculation

From 5 to 25 March of 2020, I inoculated wild *E. japonica* female flowers with either *M. reukaufii* or *A. boissieri*. These species were the most dominant yeast and bacterial species isolated from *E. japonica* at the study site, and either yeasts or bacteria were dominant in each flower, presumably because of competition between yeasts and bacteria (Tsuji & Fukami, 2018). I isolated the two species from *E. japonica* in February and March of 2016 and stored in 15% glycerol at -80 °C until 21 February of 2020. The strains I used have been sequenced; on the DNA Data Bank of Japan (DDBJ) database, the accession numbers for the *M. reukaufii* and *A. boissieri* isolates are LC333485 and LC333520, respectively (Tsuji & Fukami, 2018). Each flower was inoculated with approximately 5000 cells suspended in 0.5 μl H_2O, as in Tsuji and Fukami (2018). The cells were collected from a two-day-old colony that grew on tryptone soya agar (TSA) at 25°C, as in Álvarez-Pérez et al. (2021).

I conducted the inoculation multiple times during the 5 to 25 March period of 2020 using a total of 27 female trees. One branch contained one treatment, and three branches per tree were treated on the same day. I used mesh bags (mesh size: 0.4 mm) to enclose the flowers on individual branches prior to inoculation from December to January, which prevented insects from accessing the flowers and introducing additional microorganisms. I inoculated a total of 633, 587, and 527 blooming flowers with *M. reukaufii* cells suspended in 0.5 μl H_2O, *A. boissieri* cells suspended in 0.5 μl H_2O, and 0.5 μl H_2O as a control, respectively. Flowers with slightly colored petals indicated that they bloomed more than several days before the inoculation, were removed from the branches, but flower buds were left intact when inoculation was conducted. The buds were likely colonized via flower-visiting insects by the microbes I inoculated the neighboring flowers with, since the insects often walked on several neighboring flowers (Tsuji et al., 2020). After introducing flowers with the respective treatments, I removed the bags from a set of branches to allow pollinators to naturally visit the now exposed flowers, some of which were colonized by microbes.

Some branches were re-enclosed in the mesh bags immediately after inoculation to prevent insects from accessing the flowers to introduce additional microbes and remove the inoculated microbes. These bagged 126 flowers from 26 trees were used to estimate the density of viable cells in *E.*
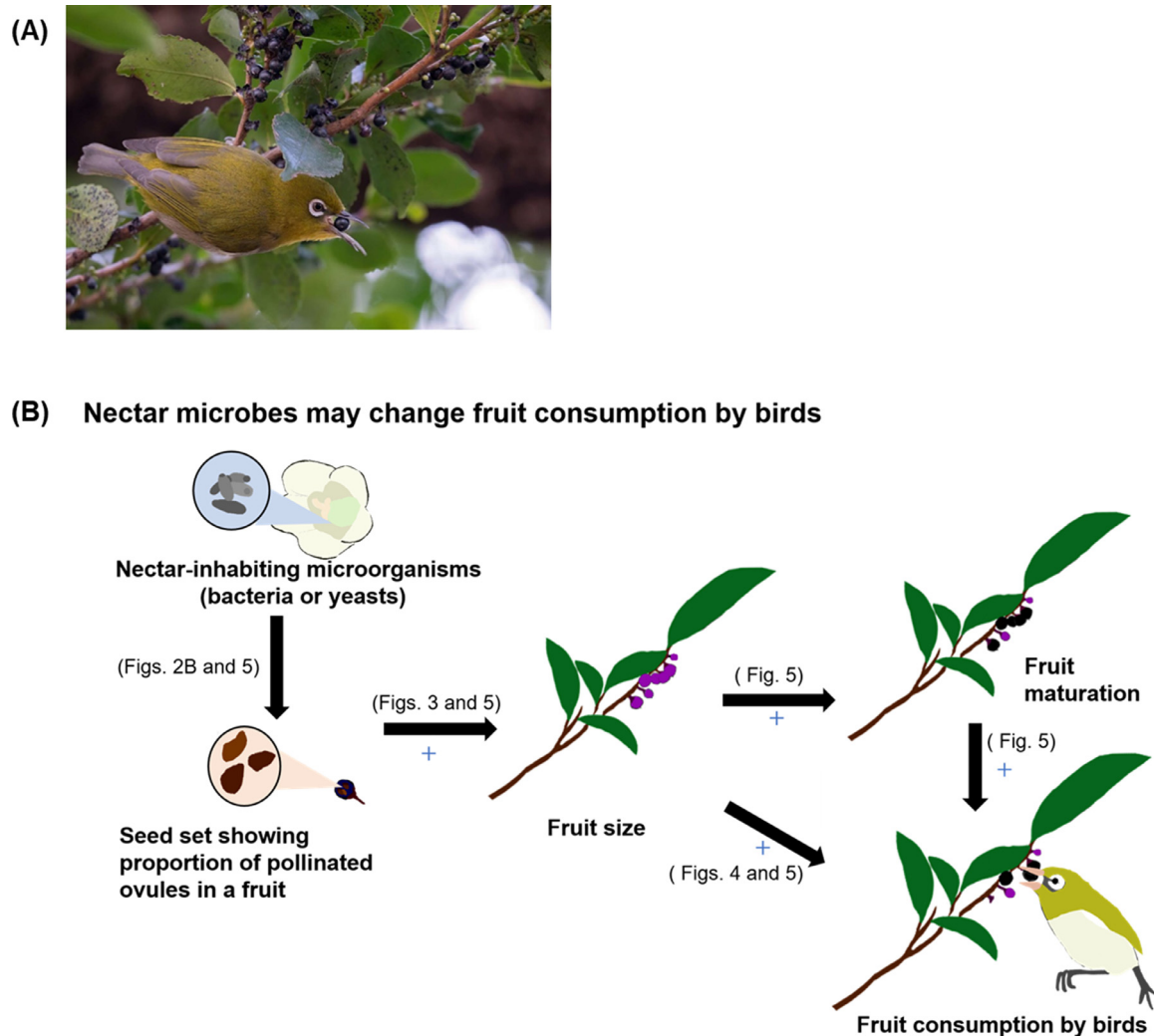
Fig. 1. Japanese white-eye *Zosterops japonica* feeding on *Eurya japonica* fruit (A). The photograph was taken by Shoji Imamura on 10-Jan-2021 at Yakushiike Park, Tokyo, Japan. Summary of this study's findings, showing the suggested possibility that nectar microbes change seed set, fruit size, fruit maturation, and fruit consumption by birds (B). Arrows show the links between nectar-inhabiting microbes, seed set (Figs. 2B and 5), where fruits with higher seed set are larger (Figs. 3 and 5), and larger fruits are preferentially removed by avian frugivores (Figs. 4 and 5) directly and indirectly via promoting fruit maturation (Fig. 5). "+" indicates positive relationship. Among microbes, yeasts decreased seed set, while bacteria had little effect on seed set (Figs. 2B and 5).

japonica nectar, as in Tsuji and Fukami (2018). Nectar was collected four days after inoculation using 0.5-μl microcapillary tubes (Drummond Microcaps). The collected nectar was diluted in 40 μl of autoclaved MilliQ water (Merck Millipore) on site and transported back to the laboratory on ice (Tsuji & Fukami, 2018). The nectar was plated on yeast malt agar (YM) with 100 mg L^{-1} chloramphenicol (antibacterial) and tryptone soya agar (TSA) with 100 mg L^{-1} cycloheximide (antifungal) for fungi and bacteria, respectively. The plates were incubated at 25°C for four days, and colony-forming units (CFUs) counted (Tsuji & Fukami, 2018). The CFUs of bacteria (Appendix A: Fig. S1) were similar to that previously observed on natural flowers in 2016 (Tsuji & Fukami, 2018), although a different medium, R2A (BD Difco) supplemented with 20% sucrose, was used then. I used TSA instead of R2A supplemented with 20% sucrose

in this study because *A. boissieri* colonies were too watery to count reliably on R2A supplemented with 20% sucrose. The CFUs of yeasts (Appendix A: Fig. S1) was also similar to that observed on YM in previous inoculation experiments (Tsuji & Fukami, 2018).

Seed and fruit set of inoculated flowers

Proportion of seed set (seed number/ovule number) and fruit set (fruit number/flower number) per branch was calculated from non-bagged inoculated flowers as follows. After the flowering season ended (May-2020, i.e., two months after the initial inoculation), I bagged the branches again to protect young fruits from frugivorous insects. After several months of bagging, I collected the branches with

bags from 21 May to 23 July of 2020. A set of branches were collected the same day. I haphazardly chose the set to collect. Since seeds and ovules in fruits were difficult to count when fruits were ripe, I collected branches when fruits were relatively young and smaller than they would have become if kept on the branch and not harvested. The data were collected for a total of 4,008 flowers on a total of 27 trees.

To examine the proportion of seed set, I counted the number of developing white seeds and dead brown ovules in young green fruits using a stereo microscope (Nikon Mini Field Microscope 20x) and tweezers.

Fruit size and fruit removal

In an earlier study at the same study site, I examined the relationship between fruit size and fruit removal by birds, I measured fruit size. This experiment was conducted in fall 2019. I used a caliper to measure the width and length of a total of 351 fruits on 12 trees. I recorded fruit color as an index of fruit maturation on 25 November 2019. Because fruit maturation could affect fruit removal, fruits were categorized into the following colors: green, blue, dark purple, and black, which ranged from immature to mature. On 24 December 2019, I recorded whether the measured fruits were still on the branch to estimate the likelihood of fruit removal by birds.

To record birds that consumed fruits at the site, I placed a time-lapse video camera (Moultrie Wingscapes Timelapse Cam Pro) two meters from the observed plant. The recording was carried out from 3-Nov-2019 to 16-Jan-2020. The camera took 90-second movies every ten minutes (Appendix A: Video S1-S2).

Statistical analysis

To test for the microbial inoculation effects on fruit and seed set, I compared the proportion of flowers that set fruits and ovules that set seeds, respectively, among three treatments: yeast-inoculated, bacteria-inoculated, and water-inoculated (control). I used generalized linear mixed models (GLMM) with a binomial distribution and a logistic function in the lme4 package (Bates et al., 2015) and the Type II Wald Chi-square test (Langsrud, 2003) in the car package (Fox & Weisberg, 2011) in R version 4.0.2 (R Core Team, 2020). In these models, I used fruit and seed set (i.e., proportion of maturation of fruit and seed, respectively) as the response variables. I also used the three treatments as fixed predictors, and plant ID and inoculation days as random effects. This GLMM was weighted by the total number of flowers and ovules, respectively. Following the GLMM, to compare fruit or seed set among the inoculation treatments, I used pairwise contrasts using the packages lsmeans (Lenth, 2016) and multcomp (Hothorn et al., 2008).

To examine how seed set was related to fruit size and whether microbial inoculation can directly affect fruit size, I used GLMMs with a Gaussian distribution. In these models, I used fruit size, i.e., estimated fruit volume (width^2 × length × 3.14 / 6), as the response variable. To examine the effect of seed set, I also used seed set, date, and their interaction as fixed predictors and plant ID as a random effect. To test the effect of inoculation, I used the three treatments, date, and their interactions as fixed predictors, and plant ID and inoculation days as random effects.

To determine whether fruit size was correlated with the proportion of fruit removal, I used a GLMM with a binomial distribution and a logistic function. In this model, I used the presence of fruits (presence or absence) as the response variable, fruit size as fixed predictors, and plant ID as a random effect.

To confirm all the single analyses done before, I did path analyses using piecewiseSEM (Lefcheck, 2016) and lme4 (Bates et al., 2015) packages in R (see , Perez-Alvarez et al., 2018). I first hypothesized that the microbial inoculation treatment (i.e., yeast inoculation, bacterial inoculation, and control) would directly change seed set, and directly and indirectly change fruit size via changing seed set. In the model with Gaussian distribution, I used seed set as a response variable and the inoculation treatment as a predictor. I also used fruit size as a response, and seed set and the inoculation treatment as predictors. I next hypothesized that fruit size would positively affect fruit consumption directly and indirectly by changing fruit ripeness. The fruit ripeness was ranked based on its color. In the model with a binomial distribution, I used fruit consumption as a response variable and seed set and fruit size as predictors. I also used fruit color as a response variable and seed set as a predictor in the model with a poisson distribution. I, at last, hypothesized that the microbial inoculation treatment would directly change fruit set. In the model with Gaussian distribution, I used fruit set as the response variable and the inoculation treatment as a predictor. In these three path analyses, I used plant ID as a random factor.

Results

Inoculation with yeasts and bacteria led to different seed set and fruit set

Experimental inoculation of flowers changed fruit set (χ^2 =24, P<0.0001) and seed set (χ^2 =105, P<0.0001). Mean fruit and seed set of flowers that were inoculated with bacteria was, respectively, 2.4% and 4.1% higher than that of flowers inoculated with yeasts (z=-2.7, P<0.05 and z=-9.1, P<0.0001, respectively; Fig. 2, Appendix A: Table S2-S3).

Higher seed set was associated with larger fruit size

Fruit size was positively correlated with seed set (estimate of coefficient=1.1, χ^2=2966, P<0.0001, Fig. 3, Appendix A: Table S4). Fruits with low seed set were consistently small,
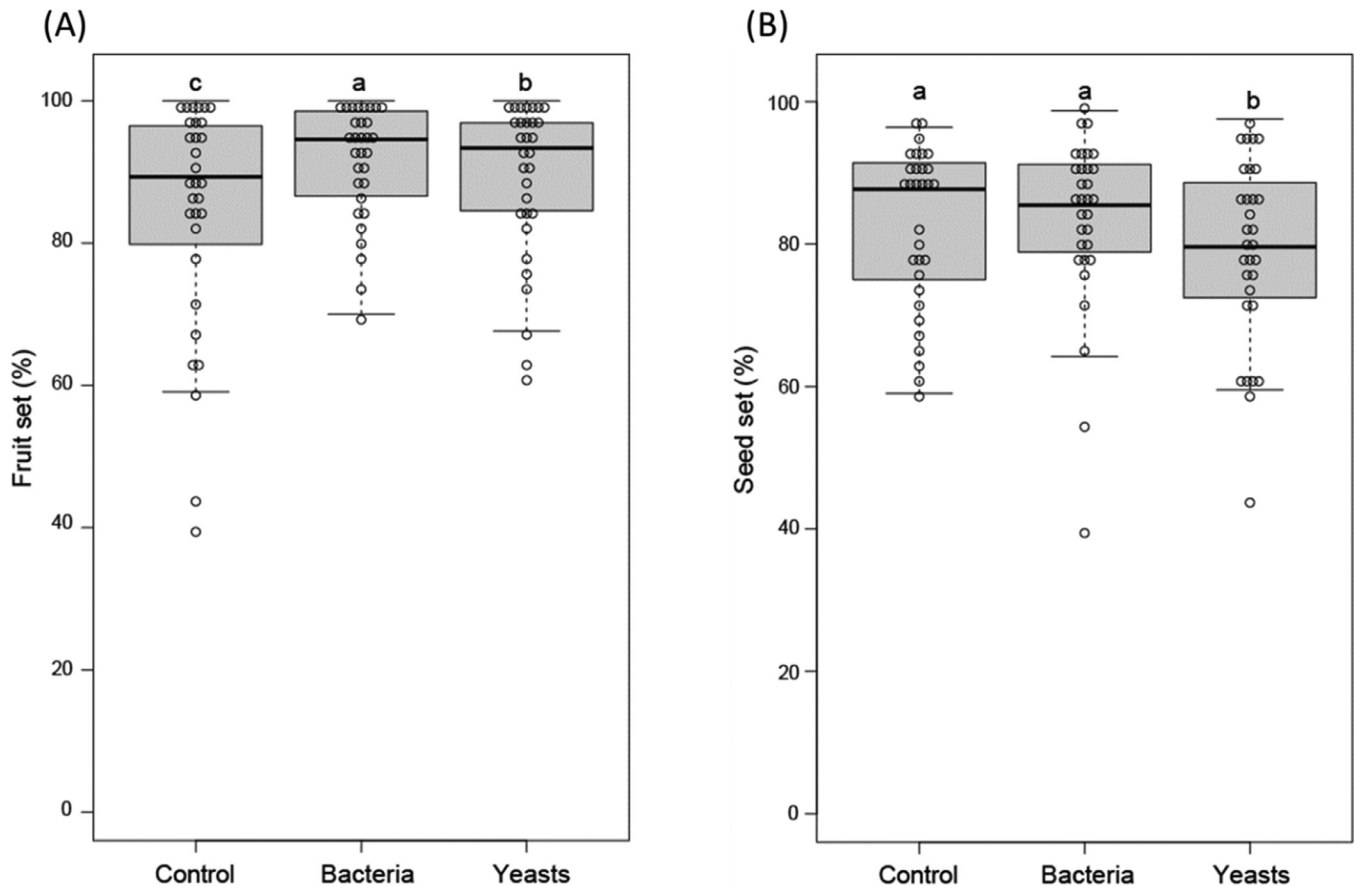
Fig. 2. Fruit set of flowers inoculated with water as a control, bacteria, and yeasts (A), and seed set of the three treatments (B). Different letters represent significant differences between groups, as determined by the post-hoc test. Treatments significantly affected fruit set and seed set (χ^2 =24 and 105, P<0.0001, <0.0001, respectively).

whereas many fruits with higher seed set were larger, especially later in the season when fruits were closer to maturation (Fig. 3). Mean fruit size was 20 and 29 mm^3 early and late in the season, respectively.

Larger fruits were more likely to be taken from the branches

Larger fruits were more likely to disappear from branches (estimate of coefficient=0.04, χ^2=17, P<0.0001, Fig. 4). During the fruiting season, the camera captured frugivorous birds, *Zosterops japonica, Hypsipetes amaurotis*, and *Phoenicurus auroreus,* removing and consuming fruits from the tree (Appendix A: Table S1, Videos S1-S2). Few fruits were found on the ground, and a few videos showed birds dropping fruits.

Yeasts and bacteria could, directly and indirectly, affect fruit size and subsequent fruit consumption by birds

Experimental microbial inoculation into flowers changed fruit size ($\chi2$ =17, P=0.0002), and yeasts decreased the size

(Appendix A: Table S5). Furthermore, the path analysis showed that yeasts, directly and indirectly, decreased fruit size via decreasing seed set consistent with the single analyses (Fig. 5). The path analysis also suggested that bacteria slightly increased seed set and slightly decreased fruit size, though the path coefficients of bacteria were similar to those of control (Fig. 5). The results of Shipley's test of d-separation supported the causal assumptions in the path model, indicating that they provided a good fit to the data (Fisher's C = 0.87, df = 2, P = 0.65). Another path analysis showed that fruit size affected fruit consumption by birds directly and indirectly via affecting fruit maturation (Fig. 5, Fisher's C = 0, df = 0, P = 1). The last path analysis supported the result of the single analysis that the fruit set of flowers inoculated with bacteria was higher than those inoculated with yeasts (Fig. 5, Fisher's C = 0, df = 0, P = 1).

Discussion

Taken together, the results suggest that nectar-inhabiting microorganisms may indirectly influence plant-frugivore interactions by changing pollination, seed set, and fruit size (Fig. 1B). Although more work is needed to definitively
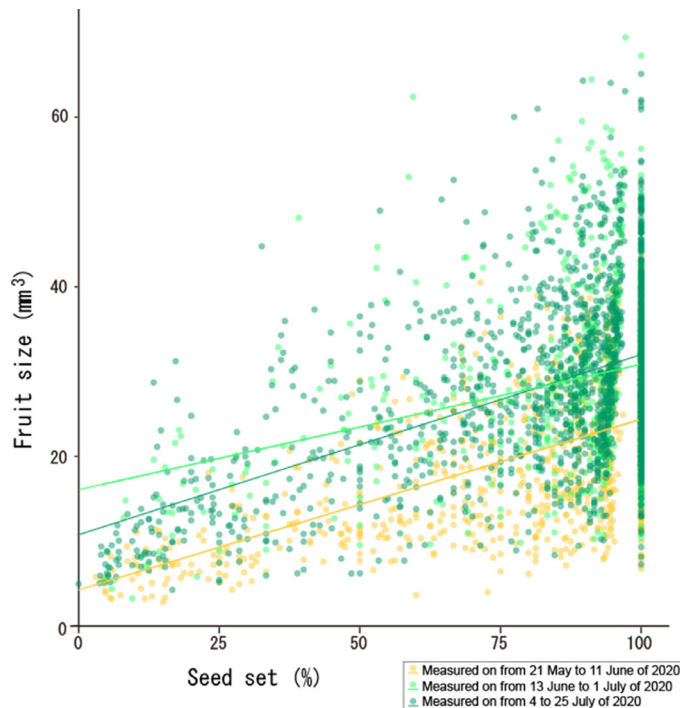
Fig. 3. Relationship between seed set and fruit size. Yellow, yellowish green and green circles indicate fruit size measured during three periods: from 21 May to 11 June of 2020, from 13 June to 1 July of 2020, and from 4 to 25 July of 2020, respectively.

establish causal links from seed set through fruit size to frugivory, the findings from this study point to the previously unrecognized possibility that nectar microbes affect not just seed set and seed mass (Herrera et al., 2013; but see Yang et al., 2019) by affecting pollinator preference (Vannette et al., 2013; Schaeffer & Irwin, 2014) and pollen germination (Eisikowitch et al., 1990; Christensen et al., 2021), but also seed dispersal by frugivores.

Overall, effect size was small in this study, with just a 4.1% change in seed set, on average, as a result of yeast and
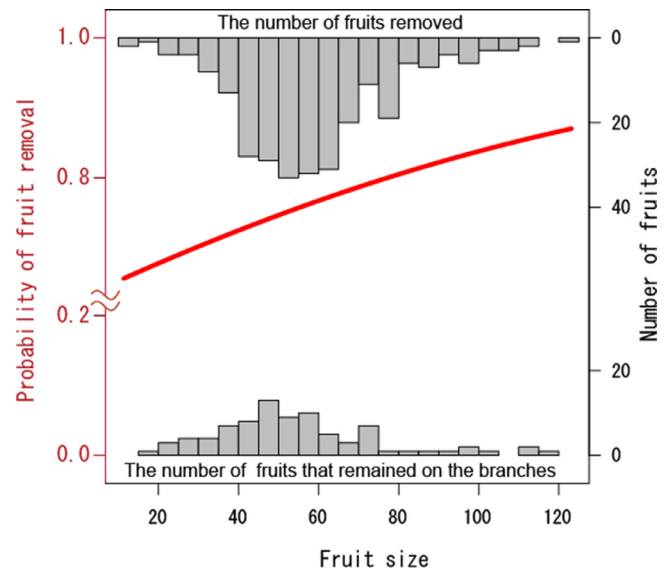

Fig. 4. Likelihood of fruit removal as a function of fruit size. The upper histrogram shows the number of fruits removed and the lower histogram shows the number of fruits that remained on the branches. Red curve shows the logistic regression curve as an index of probability of fruit removal, suggesting that birds prefered larger fruits.

bacterial inoculation (Fig. 2B). However, I suggest that the biological significance of the role of nectar microbes on frugivory may have been underestimated by the results presented here for the following reason. My data indicate that the effect size on the seed set is context-dependent (Appendix A: Fig. S2), suggesting that the effect could have been larger if we focused on flowers blooming when pollinators were not so abundant as to overwhelm the microbial effect. In this study, I calculated proportion of seed set from untreated flowers as an index of pollinator abundance on each trees, and the proportion was 80% on average (Appendix A: Fig. S2). Previously, I found that seed set could vary from year to year. In one year, 2013, for example, it was, on
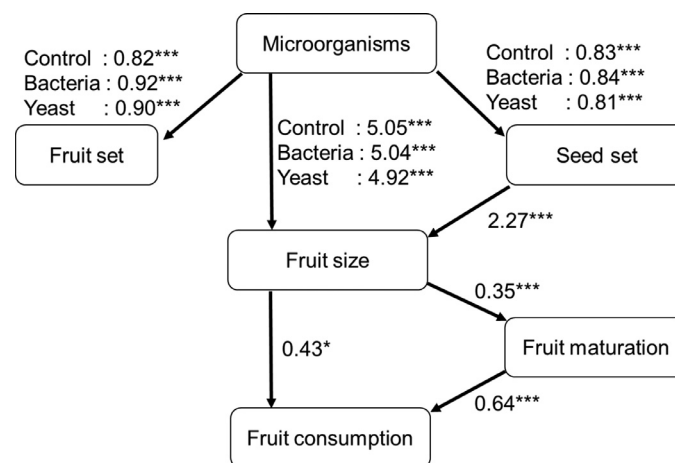

Fig. 5. Result of path analyses. Arrows represent causal relationships with significant standardized path coefficients and the number along the arrows are path coefficients (* and *** indicate p < 0.05 and 0.001, respectively).

average, 68% (Tsuji & Ohgushi, 2018; Tsuji et al., 2020). My analysis indicates that the differences of the seed set between yeast and bacterial colonization could have been greater if pollinators were less abundant (Appendix A: Fig. S2). It is not clear why this is the case, but one possibility is that pollinators can afford to have strong preference for bacteria-colonized flowers over yeast-colonized flowers when competition for nectar is not intense. Whatever the reason, my data (Appendix A: Fig. S2) suggest that yeasts would cause a reduction in seed set as large as 15% if seed set was 68%, the observed level of seed set in 2013.

Seed set was positively related to fruit size (Figs. 3 and 5), and fruit size was also positively related to fruit maturation (Fig. 5, Appendix A: Fig. S3). In some plant species, increasing seed number changes hormones and fruit development: more seeds often enlarge fruit size in apples, kiwifruits, tomatoes, etc. (Picken, 1984; Howpage et al., 2001; Eccher et al., 2014) and promote fruit maturation in apples, grapes, downy serviceberries, etc. (Cawthon & Morris, 1982; Gorchov, 1985, 1988; Obeso, 1993; Buccheri & Di Vaio, 2005; but see Gouthu & Deluc, 2015). Like these plant species, higher seed set of *E. japonica*, which suggests a larger amount of seeds in a fruit, would change hormones, enlarge fruits, promote maturation, and accelerate subsequent fruit consumption by birds. The differences in fruit maturation might affect the timing of fruit consumption and following seed dispersal. Examining whether slight differences in seed set will change fruit size, maturation, and following plant reproductive success may be interesting.

The mechanism by which yeasts and bacteria changed seed set and fruit set remains unclear and requires further investigation. The finding that bacteria caused higher fruit set than yeasts, and yeasts not bacteria decreased seed set (Fig. 2) is somewhat puzzling as this result appears to contradict earlier reports that indicated that bacteria, not yeasts, reduced pollinator visitation, seed set, and fruit set (Vannette et al., 2013; Good et al., 2014; Schaeffer & Irwin, 2014; Yang et al., 2019; but see Herrera et al., 2013; Rering et al., 2021). Though different plant species would have different flower-microbe-pollinator interactions, and many reasons simultaneously cause the differences in microbial effects on pollination, one reason may have to do with the type of pollinators involved. Previous work was conducted on plants pollinated by bees or birds, whereas *E. japonica* is mainly visited by Diptera, such as green bottle flies and flesh flies (Tsuji & Ohgushi, 2018), which often gather on carcasses and dung. These flies are attracted to fetid volatile compounds emitted from certain flowers (Jürgens et al., 2006, 2013; Shuttleworth & Jhonson, 2010; Urru et al., 2011; Zito et al., 2013, 2015). As nectar yeasts and bacteria can change floral scent in ways that affect pollinator visits (Rering et al., 2017, 2020, 2021; Schaeffer et al., 2019; Cusumano et al., 2022; Martin et al., 2022), the differential response of different pollinators to microbially modified floral scent might explain why bacteria increased fruit set compared to yeasts, and yeasts reduced seed set compared to bacteria and control

in this study. Another possible mechanism behind the negative effect of yeasts compared to bacteria on fruit and seed set is inhibition of pollen germination by yeasts, as previously suggested in the common milkweed, *Asclepias syriacal* (Eisikowitch et al., 1990), or promotion of pollen germination by bacterium *A. boissieri* not by yeast *M. reukaufii* (Christensen et al., 2021). Further experiments on visitation rate by pollinators, pollen deposition, and pollen tubes are needed to test these possibilities.

Microorganisms could mediate plant-pollinator interactions via changing flower traits (Vannette, 2020) and could directly affect pollen germination (Eisikowitch et al., 1990, Christensen et al., 2021). These microbial effects on pollination would vary fruit set, fruit size, and fruit maturation, which could affect the interaction between plant-frugivore, and subsequent plant reproductive success. In short, nectar-inhabiting microorganisms can affect plant reproductive success in various ways during both flowering and fruiting seasons.

Conclusions

The results presented here suggest that the effect of nectar microbes on seed set can result in changes in fruit size, which can in turn affect the likelihood of fruit consumption by birds, although the observed effect size was small. It is therefore possible that the effects of nectar microbes permeate through the multiple phases of plant reproduction, from seed set to seed dispersal by frugivores—a possibility that has rarely been considered, but seems worthy of further investigation given the results of this study.

Data availability statement

The data set can be found at figshare (https://doi.org/10.6084/m9.figshare.22715029.v1)

CRediT authorship contribution statement

Kaoru Tsuji designed the study, collected the data, performed the data analyses, and wrote the manuscript.

Declaration of Competing 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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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.baae.2023.04.004.

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