MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master`s Thesis

„Insights into the microbiome of *Drosophila simulans*"

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna, 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 220
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Joint-Masterstudium Evolutionary Systems Biology
Betreut von / Supervisor:	Univ.-Prof. Dr.rer.nat. Christian Schlötterer

Table of Contents

1. Abstract

Many recent studies of the microbiome used *Drosophila* as research organism because of its simple microbiome compared to humans. A lot is known about the influence of microorganisms on their host's behavior and growth rate, as well as the host's influence on the microbial composition. This study aims to identify compositional differences between two evolved populations reared under distinct environmental conditions and uses the growth rate of multiple species to show functional diversification between strains with different evolutionary histories.

Two *Drosophila* populations from Florida, which evolved either at 10-20 °C or at 18-28 °C for 175 and 87 generations, respectively, were examined using two methods: First, colonies were grown on agar plates and those with unique phenotypes were identified and stored for further analyses. Second, the whole microbiome of these cold- and hot-evolved populations was sequenced and analyzed.

While the richness of the hot-evolved population is higher, the effective number of orders is very similar between both conditions. The abundance of the three most frequent taxa is also equal. Although these two populations resemble each other well regarding the orders that make up their microbiome, this was not the case when collected bacterial strains of three common species were grown under experimental conditions.

Two species collected from the hot-evolved flies grew significantly faster than the same species collected from the cold-evolved flies, while the opposite pattern was observed for the last species. These growth patterns could originate from the bacteria's adaptation to the host's environment or represent the versatility of strains belonging to the same bacterial species. Since the relative abundances were similar for both conditions, the varying growth patterns might suggest a difference in the initial establishment of the microbiome. Future studies of the microbiome and especially its establishment should take into account that strains of the same species do not share the same growth rate across host populations, and it is very likely that they differ in several physiological processes.

2. Introduction

The microbiome influences its host in a variety of ways from very specific interactions like the bioluminescence of squids [1] to more general functions necessary for development, metabolism and immunity in animals [2]–[5]. Temperature has been shown to affect microbial composition across many species [6]. In humans the gut microbiome is made up almost entirely of bacteria (99.1 %) [7] and is important for studying many diseases like inflammatory bowel syndrome, diabetes and cancer [8].

A fair amount of research has been done on the microbiome of fruit flies (*Drosophila*). It is not surprising that *Drosophila* was chosen, since fruit flies offer many general advantages such as short generation time (ca. 12 days from egg to fertile imago), high number of offspring, easy husbandry and large variety of experimental tools [9]. What makes them especially valuable for microbial studies is their relatively small microbiome (1–30 bacterial taxa measured in various *Drosophila* species), when compared to vertebrate microbiomes (> 500 taxa) [3][10][11]. Even natural populations of *Drosophila* host microbial communities of only 100-200 Operational Taxonomic Units (OTUs) [12]. In contrast to previous studies Wong et al. (2013) described that out of 21 populations, comprised of ten *Drosophila* species (18 laboratory and three field-collected samples), all but one species displayed more than 30 bacterial taxa in their microbiome. They reported a maximum of 318 OTUs in a laboratory strain and 178 OTUs in a field sample. This could be attributed to laboratory conditions or other *Drosophila* species being used than in earlier studies. It is important to know that, even in this data set of high numbers of OTUs, the species that will be used in the following experiments, *Drosophila simulans*, had a low number of only 36 OTUs. [12]

The microorganisms found in fruit flies can be put in three categories: gut microbiota, external microbiota and endosymbionts. The most prominent of the latter is *Wolbachia*, which makes up most of the bacterial load in infected fruit flies [13][14]. It is distributed via vertical transmission from one generation to the next [15]. One might think that the gut and external microbiome should be very different, because of the amount of oxygen and other parameters, but they have been shown to consist of similar organisms, even though the frequencies of the taxa did not match [16].

Since most of the microbiome of the fruit fly is concentrated in the gut, it is important to know the gut's physiology. It consists of multiple organs similar to the human digestive tract, but does not feature an extensive anoxic region [15]. Although this could lead to the conclusion that only aerobic bacteria make up the gut microbiome, it has been shown that aerotolerant anaerobic species of the genus *Lactobacillus* live inside and outside of fruit flies [16].

In comparison to the human digestive system, the fly gut provides a rather unstable environment for bacteria due to the shedding of the cuticle lining at each larval moult and in the adult fly [11]. Microbes might also be less strongly associated with the fruit fly due to its short life span. To maintain a persistent microbiome, flies need to take up bacteria from their food source and then either defecate or regurgitate them onto fresh food. This process distributes the microorganisms to other flies in the group as well as to the next generation, that feeds on the bacteria-laden sustenance as larvae. When studying the microbiome of *Drosophila* in laboratories, stocks need to be kept on the same food source for 3-4 days, because switching them to new food every day would reduce their bacterial load. [17]

Using this knowledge, Pais et al. (2018) tested if there were stable bacterial communities in the gut of laboratory and natural populations of *D. melanogaster* by switching them to a new vial twice a day. After 10 days it became apparent that part of the microbiome of the natural

population was maintained, while only a tiny fraction of the bacteria found in the laboratory population persisted. They further reported that *Acetobacter cibinongensis*, *A. thailandicus* and *Lactobacillus brevis* proliferate in the gut, while other bacteria seem to grow only in the food and need to be ingested again to increase bacterial levels. [18]

Most studies have focused on how microbiota shape fruit fly adaptation ranging from tolerance of new food sources or environmental conditions to changes in anxiety-like behavior, locomotion and perhaps mate choice [4][19]–[23]. Although the influence of the host and its surroundings on the composition of the microbiome has also been discussed often [10][24]–[26], the effect of temperature has only been studied over short time periods [27][28]. This study tests if the microbial composition changes due to the preference of some bacteria for lower or higher temperatures [29].

The Institute of Population Genetics (IPG) has maintained cold- and hot-evolved fly populations (fluctuating temperature regime: 10-20 °C, 18-28 °C) for 87 and 175 generations, respectively, that have been intensively studied [30]–[36]. These populations were used in culturing experiments and for 16S rRNA sequencing to study functional diversification under various growth conditions e.g. media and oxygen content (see Fig. 1). Furthermore, growth experiments were performed on multiple strains of *Acetobacter*, *Lactobacillus* and *Leuconostoc* to test the hypothesis, that these species also adapted to the temperature and therefore differed across the two evolutionary conditions.

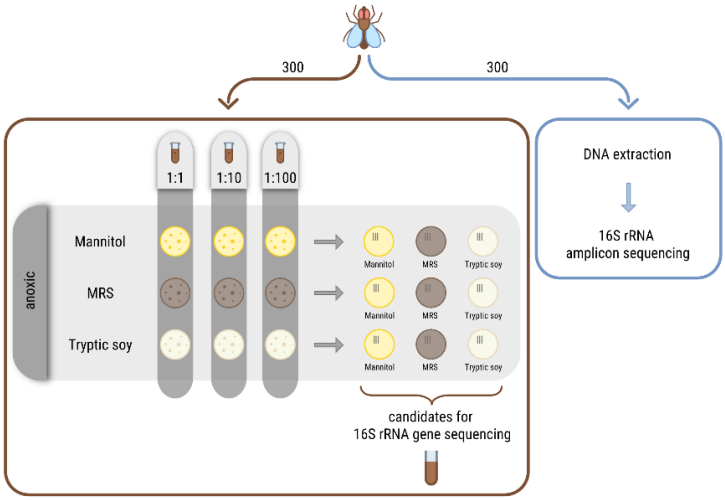

Fig. 1: Overview of the approaches used to identify compositional differences between the cold- and hot-evolved fly populations: Colonies were collected, sequenced and stored for later experiments (brown). 16S rRNA sequenced was used to determine the microbial composition (blue).

3. Results

3.1 Cultured microbiomes of *D. simulans* populations

Since no previous data on the fly populations of the IPG is available, this study generated a general overview by culturing homogenates of *D. simulans* populations originating from three different regions (South Africa, Portugal and Florida) under various conditions. To avoid excluding bacteria because of the culturing medium, dilutions were grown on three plate media (mannitol, MRS, tryptic soy) under various conditions. Colonies from each plate type were selected and put on all three plate media to select the most diverse set of colonies by detecting differences in growth, color and shape among all three media. Furthermore, cultures were grown under an- and oxic conditions, since both an- and aerobic microbes can be found in fruit flies [16]. The 250 bacterial strains (~30 per experiment) collected during this first look into the culturable microbiome of *D. simulans* populations of the IPG were amplified using two 16S rRNA gene primers: "670 bp" and "16S long" (see 6.1). The 16S long primer set was used for samples that did not produce a fragment using the 670 bp primers, mainly *Acetobacter* (see 3.2.1). After Sanger sequencing the 16S rRNA gene fragment, the species of the colonies were identified using blastn and stored for future studies (e.g. the growth experiments described in 3.3) [37]. Tables featuring all cultured microbiomes can be found in the *Appendix*. A phenotypic overview of the colonies can be found at the end of this chapter.

Influence of the temperature on microbial composition

To test whether incubating cultures at 37 °C, a temperature often used in microbiome studies [13][17][38][39], introduced an unwanted selection criterion, a South African fly population reared at 15 °C was cultured at 20 °C and 37 °C under oxic conditions. As seen in Table 1, *Leuconostoc* seems to be unaffected by temperature changes, but other bacteria occur only at specific temperatures. To avoid the unwanted selection by temperature shown in this experiment, all cultures (except for Fig. 2: A) were grown at the same temperature regime that was used while rearing the flies.

Table 1: Number of colonies found in South African *D. simulans* cultured at either 20 °C or 37 °C on tryptic soy plates under oxic conditions. 16S rRNA fragments were used for identification via blastn (see 6.1).

	20 °C	**37 °C**
Leuconostoc mesenteroides* and *L. pseudomesenteroides	4	3
Enterobacter ludwigii* and *Citrobacter freundii	4	0
Enhydrobacter aerosaccus* and *Moraxella osloensis	2	0
Staphylococcus hominis	0	2
Lactobacillus plantarum	0	3

Influence of oxygen on microbial composition

Three populations were used for testing if the oxygen content affects the composition of the culturable microbiome by growing cultures under an- and oxic conditions. Two cultures show almost no differences between the an- and aerobic microbiome (see Fig. 2: A and C). In population B only two species are found under both conditions: *E. faecalis* and *L. plantarum*. However, since population B is a subsequent generation of population A cultured at another temperature, the results of population B should be similar to population A and thus the experiment should be repeated to obtain more data points.

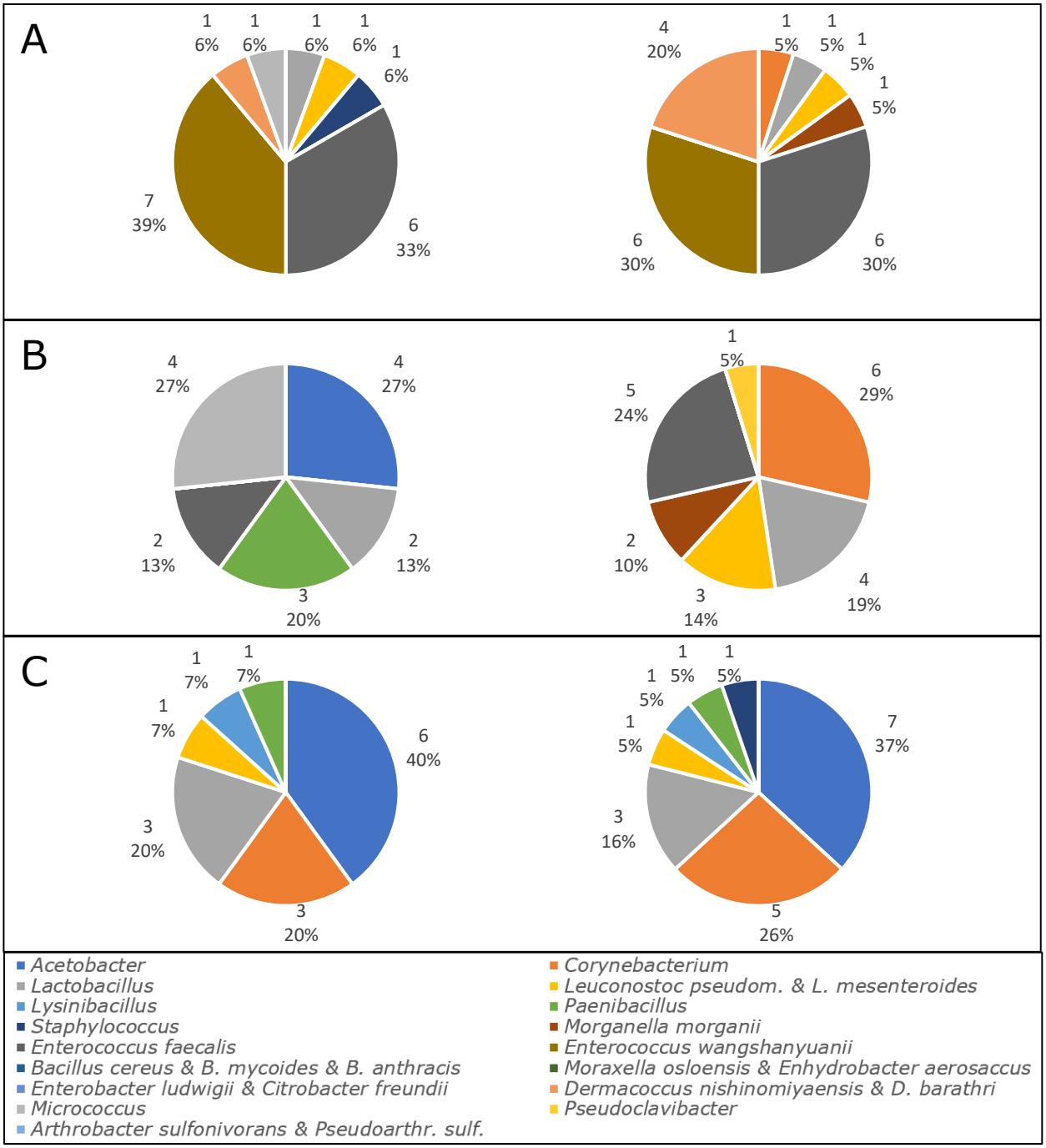

Fig. 2: Cultured microbiomes of three populations (A, B and C), aerobic (left) and anaerobic (right): A: Portuguese population reared at 18-28 °C cultured at 37 °C on mannitol and tryptic soy B: Portuguese population reared at 18-28 °C cultured at 18-28 °C on all three media C: Floridian population reared at 18-28 °C cultured at 18-28 °C on all three media

Long-term influence of temperature on microbial composition

Two Floridian *D. simulans* populations maintained at fluctuating temperature regimes between 10-20 °C and 18-28 °C for 87 and 175 generations, respectively, were used for culturing experiments to find out if the microbiome of these two populations evolved differently. Since the variation between the an- and aerobic microbiome of the Floridian flies depicted in Fig. 2: C (which is a different replicate and earlier generation of the hot-evolved population) was low, only anoxic conditions were used to study the microbiome of the hot- and cold-evolved flies. The hot-evolved fly population (replicate 4) was studied at generation F175 and F176 (see Fig. 3). The microbiome appears stable over the course of one generation: Only 17 % (F175) and 23 % (F176) of the microbiome occur in only one generation. Both microbiomes also resemble the anaerobic microbiome of the related Floridian flies shown in Fig. 2: C, although the second most abundant genus changed from *Corynebacterium* to *Leuconostoc*.

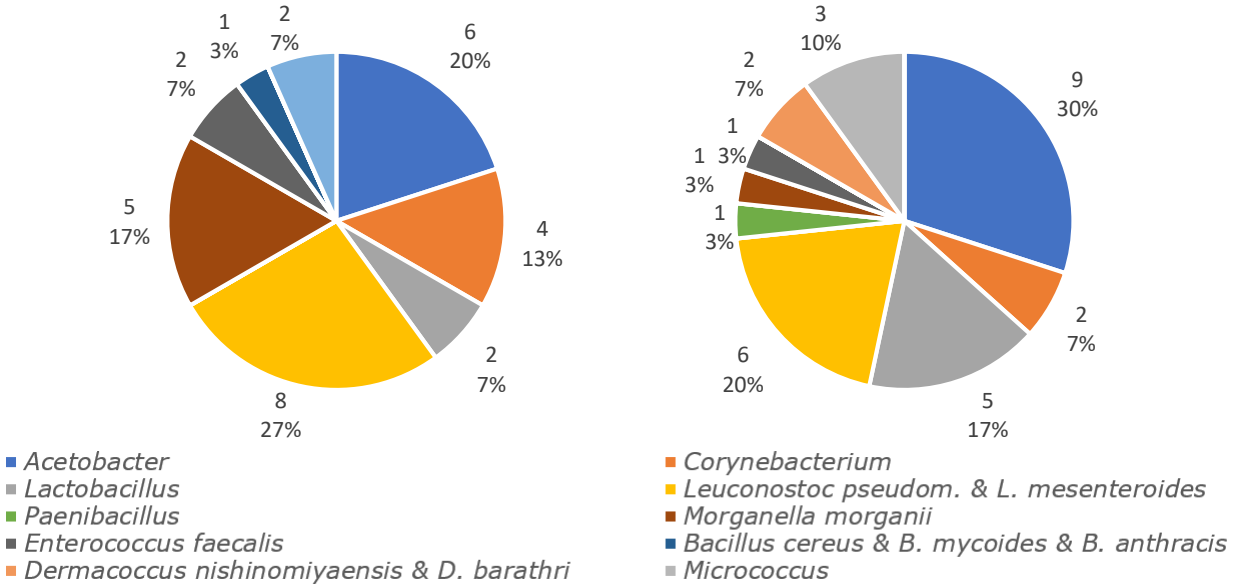

Fig. 3: Hot-evolved Floridian fly population at generation F175 (left) and F176 (right) cultured at 18-28 °C on all three media under anoxic conditions

Cold-evolved flies of generation F87 collected before and after the egg lay were also cultured using their fluctuating temperature regime under anoxic conditions to examine the expected loss of bacteria resulting from additional changes of the food source [17]. The sample before the egg lay depicted in Fig. 4 has a smaller number of taxa compared to the hot-evolved microbiomes shown in Fig. 3. Out of the six taxa present in the cold-evolved microbiome before the egg lay only three also occur in both hot-evolved generations. The number of taxa decreased even more after the egg lay, which might be related to the change of the food source during the egg lay. However, three out of the four most abundant genera are present in all samples: *Acetobacter*, *Lactobacillus* and *Leuconostoc*. These genera were studied further in growth experiments to find out if their phenotypes differed depending on the flies they originated from (see 3.3 Growth experiments). The cultured samples suggest that the hot-evolved flies have a microbiome with a diverse spectrum of bacteria, while the cold-evolved population consists of few taxa. To test if this holds true for the whole, culturable and unculturable, microbiome these populations were analyzed using 16S rRNA sequencing (see 3.2. 16S rRNA sequencing), which should deliver more robust results than these cultures that have a small sample size; and thus, experience high stochasticity.

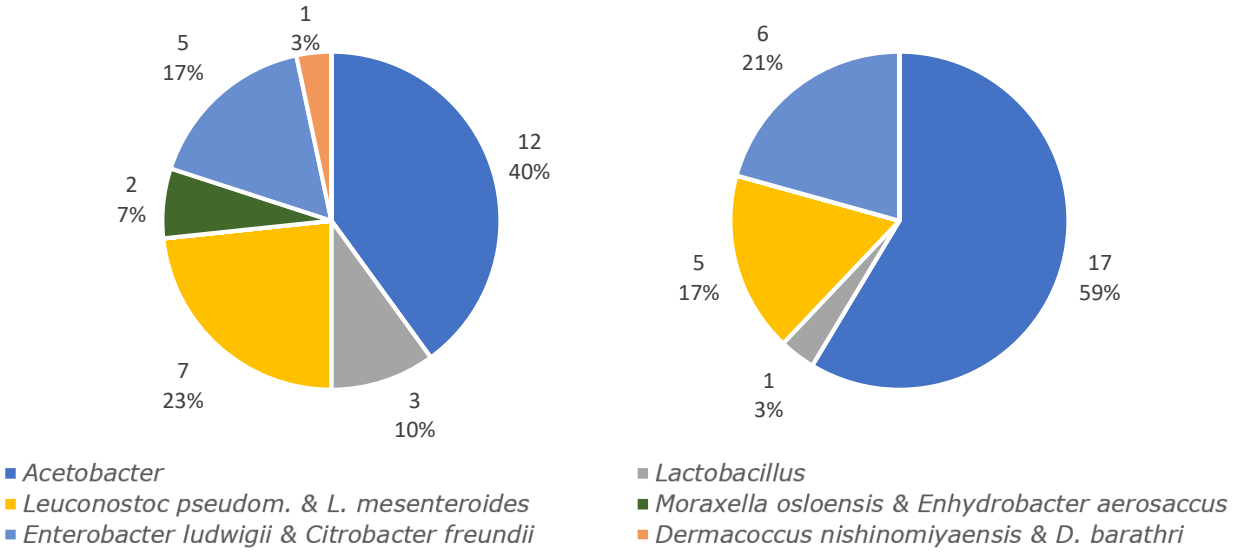

Fig. 4: Cold-evolved Floridian fly population at generation F87 before (left) and after (right) the egg lay cultured at 10-20 °C on all three media under anoxic conditions

Overview of anaerobic microbiomes cultured at the flies' temperature regime

The pie charts in Fig. 5 demonstrate the variability between different fly populations raised in the same laboratory on the same food source and even the same temperature regime (excluding the cold-evolved Floridian population) [10][12]. Only two out of the three most abundant genera were present in all samples: *Lactobacillus* and *Leuconostoc*. However, the most abundant genus, *Acetobacter*, is missing from the Portuguese population. Although the number of colonies collected for the Portuguese and the Floridian replicate 5 population were only ~2/3 of the other populations, they still achieved an equal or higher number of taxa than the cold-evolved Floridian population before the egg lay. Furthermore, in all populations except for the cold-evolved population (before and after the egg lay) *Corynebacterium* could be found, while a genus identified as both *Enterobacter* and *Citrobacter* could only be found in the cold-evolved population. These results indicate that some bacteria prefer specific temperatures and that hot-evolved fly populations in general have a richer microbiome than cold-evolved populations.

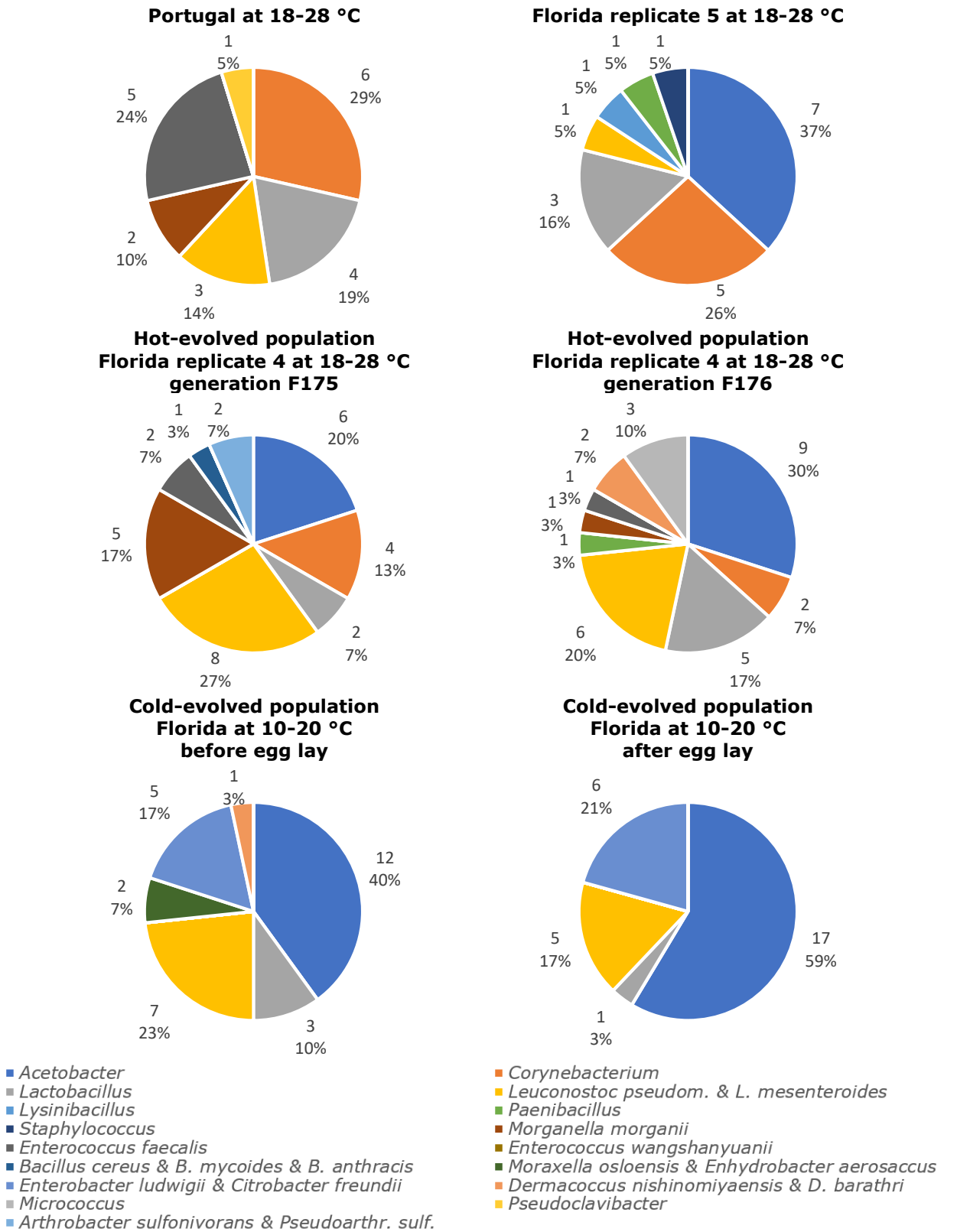

Fig. 5: Pie charts of all anaerobic microbiomes obtained by culturing at the temperature regime used for rearing the respective fly populations

Phenotypic diversity of colonies

After culturing experiments for various *D. simulans* populations were performed to take a first look at their culturable microbiome, the phenotypic traits of the colonies, that were identified and stored, were analyzed. Knowing which color, surface and growth behavior certain bacteria express on the three media will enable preliminary identification of colonies in future studies. It could also help detect contamination e.g. *Dermacoccus*, which was found in cultures made from homogenate of Floridian flies but was not present in the amplicon sequencing data (see 3.2) of the microbiome of these flies and thus might originate from contamination.

Combining all the data of the stored bacteria revealed some taxa that followed specific color and growth patterns across the three media, e.g. *Enterococcus faecalis* (see Table 2). Various bacteria show a range of patterns, for example *Lactobacillus*. The most diverse species is the highly abundant *Acetobacter indonesiensis*. This species' colonies appeared beige or white and never showed any distinguishable growth patterns. This large variety of phenotypes among *A. indonesiensis* indicates that the bacteria assigned to this species might act very different from each other.

Table 2: Phenotypic patterns of the most abundant bacteria from all microbiomes found by culturing

	Color	**Surface**	**Growth**
Acetobacter indonesiensis	diverse		diverse
Corynebacterium	beige		all media
Dermacoccus	orange		all media
Enterococcus faecalis	off-white or beige		all media
Enterococcus wangshanyuanii	white		sometimes no growth on mannitol
Enterobacter ludwigii & Citrobacter freundii	white		only rarely growth on MRS
Lactobacillus	yellow or eggshell	sometimes uneven	all media
Leuconostoc	white	sometimes uneven	all media
***Morganella morganii* pattern 1**	prismatic, see-through	rarely uneven	no growth on MRS
***Morganella morganii* pattern 2**	diverse		all media
Paenibacillus	diverse		no growth on MRS, sometimes also no growth on mannitol

3.2 16S rRNA amplicon sequencing

3.2.1 Primer comparison

Two evolved populations of *D. simulans* reared at temperature regimes fluctuating between either 10-20 °C for 87 generations or 18-28 °C for 175 generations were examined in this study by culturing their microbiome (see 3.1 Cultured microbiomes of *D. simulans* populations). To obtain a better picture of the whole, culturable and unculturable, microbiome 16S rRNA amplicon sequencing was used to identify bacteria. In addition to the IPG's standard primers, the primer pairs "670 bp" and "16S long" (see *Materials and Methods*) were tested on the cold-evolved generation F87 before and after the egg lay and the hot-evolved generation F175 before the egg lay. The analysis of multiple generations of the cold- and hot-evolved microbiome obtained via 16S rRNA sequencing can be found under 3.2.3.

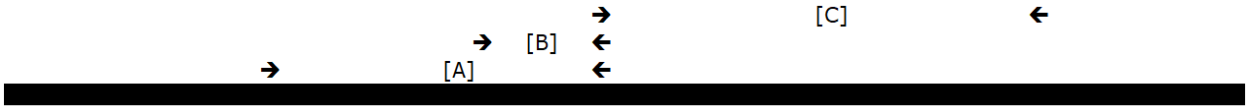

Fig. 6: Depiction of the 16S rRNA gene and the positions of all three primer sets: [A]: region amplified by the 670 bp primer set; [B]: region amplified by the 16S long primer set; [C]: region amplified by the standard primer set

The number of reads obtained via the 16S long and 670 bp primers are comparable (see Table 3). However, the standard primer resulted in a smaller library size.

Table 3: Overview of the microbiome samples analyzed at the order level using the three primer pairs: 670 bp, 16S long and IPG's standard

Order	Cold						Hot		
	F87						F175		
	1			2			1		
	670 bp	16S long	stand-ard	670 bp	16S long	stand-ard	670 bp	16S long	stand-ard
Rickettsiales	0	13443	13371	0	17660	12482	2	17130	16886
Enterobacterales	12412	1703	314	7624	519	18	1316	69	3
Rhodospirillales	0	4545	1446	2	9688	2015	0	1983	527
Lactobacillales	1743	346	140	5045	203	53	9012	116	39
Pasteurellales	21	1640	0	0	1	0	0	0	0
Actinomycetales	759	299	90	86	0	0	89	25	0
Oceanospirillales	140	15	0	779	12	0	80	0	0
Pseudomonadales	36	2	5	343	0	12	398	0	5
Vibrionales	0	0	0	559	27	0	55	0	0
Xanthomonadales	134	2	8	53	0	0	211	0	1
Saprospirales	2	24	1	14	60	2	32	223	39
Bacillales	78	25	4	54	0	0	0	33	0
Alteromonadales	0	0	0	71	0	0	27	0	0
Clostridiales	0	0	0	27	0	0	53	0	0
Aeromonadales	0	0	0	42	0	0	0	0	0
Burkholderiales	0	0	1	0	0	0	36	0	1
Rhizobiales	0	1	0	0	9	1	0	22	0
Rhodobacterales	24	0	0	0	0	0	0	0	0
Bacteroidales	0	0	0	16	0	0	0	0	0
Thiotrichales	0	0	0	13	0	0	0	0	0
Flavobacteriales	0	0	0	8	0	0	0	0	0
Legionellales	8	0	0	0	0	0	0	0	0
Myxococcales	7	0	0	0	0	0	0	0	0
Ellin329	0	5	0	0	0	0	0	0	0
Erysipelotrichales	0	0	0	0	0	0	1	0	0
Planctomycetales	0	0	0	1	0	0	0	0	0
Flavisolibacter	0	0	0	0	0	0	0	1	0
Percentage of reads assigned	65.2	100.0	99.9	63.9	95.5	100.0	62.0	100.0	99.9
Library size	23575	22060	15392	23079	29513	14590	18233	19607	17517

The microbiome of all samples except for those amplified using the 670 bp primer is dominated by the endosymbiont *Wolbachia* (order Rickettsiales). The 670 bp primer set was not able to amplify Rickettsiales and obtained a far lower average for the percentage of assigned reads than the other two primers (see Table 4). Since Kraken 2 does not provide the unassigned reads, these reads were not identified further. The second most abundant order Rhodospirillales is also missing from the 670 bp data, which includes the genus *Acetobacter* that was notably not amplified by the 670 bp primer in a previous experiment (see 3.1). Lactobacillales and Enterobacterales, two very abundant orders, could be found in all samples.

Table 4: Averages of the three samples examined using all primer pairs: 670 bp, 16S long and standard

	670 bp	**16S long**	**standard**
Library size	21629	23727	15833
Assigned reads (%)	63.7	98.5	99.9
Rickettsiales (%)	0.0	69.4	89.6
Without Rickettsiales			
Richness	13.7	9.0	7.3
Rhodospirillales (%)	0.0	71.5	83.6
Lactobacillales (%)	26.2	3.5	5.2
Enterobacterales (%)	31.0	9.0	5.6

To further examine the orders Rickettsiales and Rhodospirillales, genus level data of the samples (see Table 15) was used and for each genus one species was chosen as an example. Sequence alignments were performed on the examples' 16S rRNA gene and the primer sequences (see Table 16) and revealed mutations in the region of the forward primer of the 670 bp primer set. Although mutations were also found in the 670 bp reverse primer region, these mutations should not be problematic for the amplificant, since the 16S long shares the same reverse primer and was able to amplify both genera (see Fig. 6).

The genera belonging to the orders Rickettsiales and Rhodospirillales were removed before the analysis of the remaining genera. Pearson's Chi-squared tests for count data were performed to test if the representation of each genus was equally strong among all primers (see Table 5). All comparisons were found to be significantly different.

Table 5: Pearson's Chi-squared tests for count data of the primer comparison samples (genus level)

	Cold-evolved F87 before egg lay			**Cold-evolved F87 after egg lay**			**Hot-evolved F175 before egg lay**		
***p*-value**	0.0005			0.0005			0.0005		
Bonferroni corrected *p*-value	0.0015			0.0015			0.0015		
Pairwise comparison	**670 bp vs 16S long**	**670 bp vs stand-ard**	**16S long vs stand-ard**	**670 bp vs 16S long**	**670 bp vs stand-ard**	**16S long vs stand-ard**	**670 bp vs 16S long**	**670 bp vs stand-ard**	**16S long vs stand-ard**
***p*-value**	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
Bonferroni corrected *p*-value	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045	0.0045

Most genera could only be amplified by one or two of the three primer sets or obtained much lower counts using one of the three primer sets. To examine these taxa the data set without the orders Rickettsiales and Rhodospirillales was used. First, all genera that appeared in at least two 670 bp samples were further analyzed. The frequencies of the genera in the 670 bp samples were used to calculate the binomial distribution probability and find out if the counts obtained by the other two primers were lower than expected (see Table 17). Sequence alignments were performed on example species for these taxa. Then, this process was repeated using the 16S long and standard primer frequencies as starting points (see Table 18). Out of the 17 examined genera among all primers only six have mutations in regions associated with the primers that could explain the low counts observed. Furthermore, an additional Pearson's Chi-squared test was performed using only genera that had no mutations in the 16S rRNA region and returned the same results as before (see Table 5).

3.2.2 Representation of the microbiome

In Fig. 7 the cultured bacteria (3.1) as well as microbial compositions obtained by 16S rRNA sequencing (3.2.1) using three different primers (670 bp, 16S long and IPG's standard) are shown to allow a direct comparison of the representation of the same microbiome. The genus *Wolbachia* was removed because it is not culturable and for visualization purposes. Because of the small sample size (~30) and the fact that unique phenotypes were selected during the culturing experiments, the microbiome is skewed by an overrepresentation of low frequency taxa. Although the 16S sequencing experiments had a far bigger sample size (>1 800), the representation of the microbiome was not good using one of the primers, 670 bp. Since this primer set cannot amplify the very abundant genera *Wolbachia* and *Acetobacter*, it works like a magnifying glass for the less abundant genera but does not represent the microbiome as a whole. The remaining two primers, 16S long and IPG's standard, are both good representations of the microbial composition. However, the standard primer set depicted more richness per library size (0.00047) compared to the 16S long primer set (0.00039) and should be preferred for libraries of equal size.

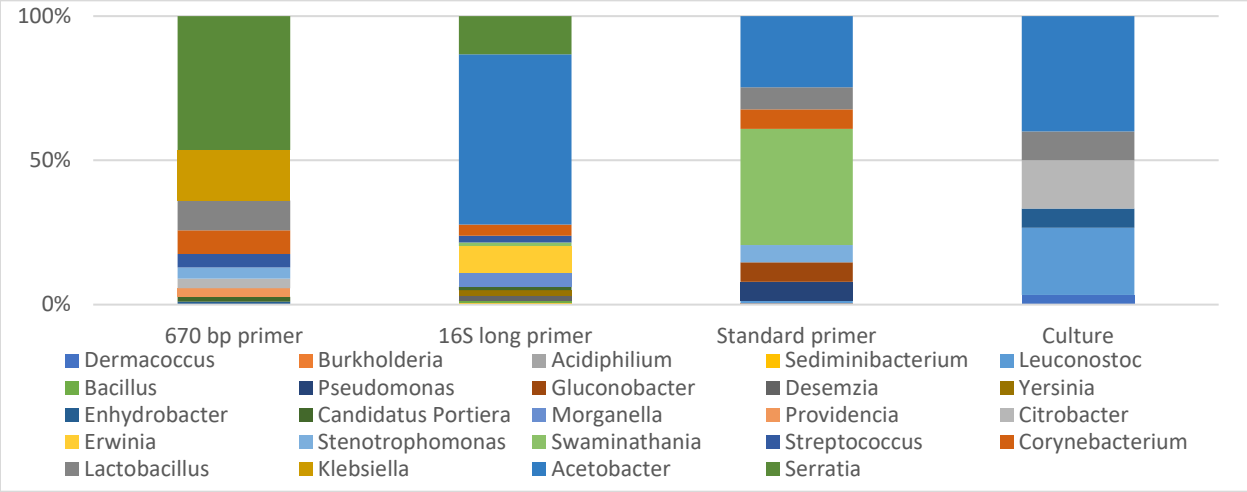

Fig. 7: Column chart of genera of generation 87 of the cold-evolved fruit flies before the egg lay: The endosymbiont *Wolbachia* is excluded for better visualization.

3.2.3 Cold- and hot-evolved microbiomes

16S rRNA sequencing experiments using only IPG's standard primer were run in parallel to the experiments comparing three primer sets (see 3.2.1). These experiments were performed to study two evolved populations of *D. simulans* reared at temperature regimes fluctuating between either 10-20 °C for 87 generations or 18-28 °C for 175 generations that were also examined in this study by culturing their microbiome (see 3.1). Additional flies of these populations were used for 16S rRNA amplicon sequencing to see if the differences between the two evolutionary conditions found in the culturable microbiome could be replicated. The microbiomes of multiple generations of the cold- and hot-evolved fly populations before and after the egg lay were sequenced to additionally study if there were large changes before and after the egg lay as well as between the generations.

The resulting libraries ranged from ~15 000 to ~21 000 reads (see Table 6). Only four orders could be found in all samples. Rickettsiales, the order *Wolbachia* belongs to, has the most assigned reads (see Table 7). The next most abundant order in all samples is Rhodospirillales, which includes *Acetobacter* and makes up ~80 % of the assigned reads (excluding Rickettsiales) on average in the cold- and hot-evolved populations. The last two orders found in all samples are Enterobacterales and Lactobacillales. No inference can be drawn for other orders because the library was not of sufficient depth.

Table 6: All microbiomes of cold- and hot-evolved fruit flies sequenced using the standard primers: "1" and "2" refers to before and after the egg lay.

Order	**Cold**					**Hot**			
	F87		**F88**		**F89**	**F175**		**F176**	
	1	**2**	**1**	**2**	**1**	**1**	**2**	**1**	**2**
Rickettsiales	13371	12482	18050	18660	16127	16886	16107	11029	15898
Rhodospirillales	1446	2015	1197	2348	1471	527	2552	5042	2977
Enterobacterales	314	18	165	286	5	3	414	33	1976
Lactobacillales	140	53	199	111	79	39	47	110	279
Actinomycetales	90	0	0	5	1	0	39	121	155
Pseudomonadales	5	12	10	16	0	5	8	26	31
Saprospirales	1	2	0	0	0	39	4	0	2
Bacillales	4	0	3	1	0	0	0	4	0
Burkholderiales	1	0	0	1	0	1	5	4	0
Xanthomonadales	8	0	0	0	0	1	0	0	0
Pasteurellales	0	0	1	0	0	0	2	0	2
Caulobacterales	0	0	0	0	0	0	0	4	0
Desulfuromonadales	0	0	0	0	0	0	3	0	1
Oceanospirillales	0	0	0	0	0	0	0	2	0
Rhizobiales	0	1	0	0	0	0	0	1	0
Clostridiales	0	0	0	0	0	0	1	0	0
Flavobacteriales	0	0	0	0	0	0	0	0	1
Thiotrichales	0	0	0	0	0	0	0	1	0
Percentage of reads assigned	99.9	100.0	100.0	99.9	100.0	99.9	100.0	100.0	100.0
Library size	15392	14590	19628	21445	17689	17517	19188	16382	21332

After removing the order Rickettsiales from each sample the microbiome was analyzed using the Wilcoxon rank-sum test on the alpha diversity parameters: richness, i.e. number of orders, and the inverse Simpson index, i.e. effective number of orders. Although Fig. 8 shows that most hot-evolved microbiomes obtained higher levels of richness (p-value = 0.06281), this might be affected by the larger library size of these samples (see Table 7) [40]. Thus, the effective number of orders, which takes the proportional abundance into consideration, is a better parameter upon which to compare the cold- and hot-evolved fly populations. Analysis of this parameter revealed no significant differences between the two evolutionary conditions (p-value = 0.7302) [41].

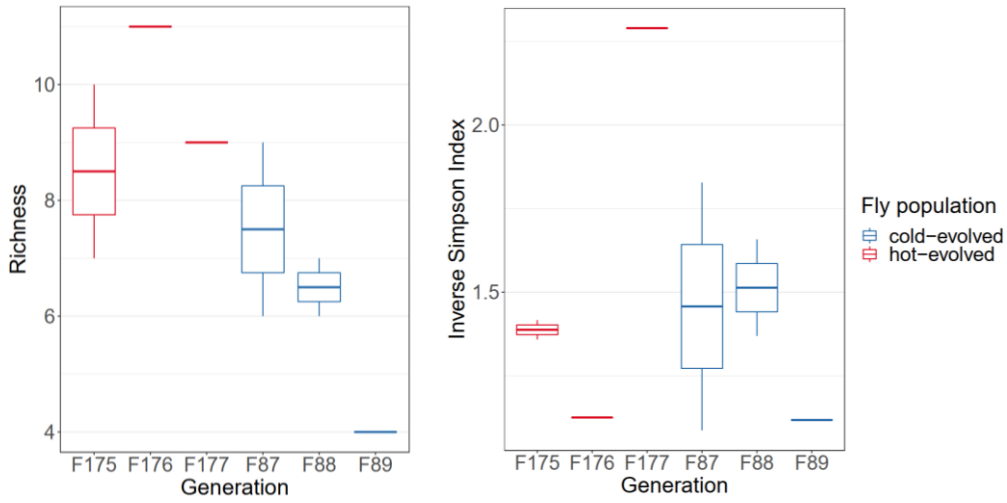

Fig. 8: Boxplots for the richness (left) and inverse Simpson index (right) of cold- and hot-evolved fruit fly populations per generation

Table 7: Average library size, fraction of assigned reads, richness, inverse Simpson Index and frequency of the most abundant taxa of 16S sequencing samples of cold- and hot-evolved flies

	Cold-evolved flies	**Hot-evolved flies**
Library size	17749	18605
Assigned reads (%)	99.9	99.9
Rickettsiales (%)	88.5	80.5
Without Rickettsiales		
Richness	6.4	9.3
Inverse Simpson index	1.4	1.5
Rhodospirillales (%)	84.3	78.8
Lactobacillales (%)	6.2	3.7
Enterobacterales (%)	7.5	12.7

Additionally, the effective number of orders was compared between samples before and after the egg lay. It decreased in the cold-evolved population but remained the same in the only hot-evolved sample. To further verify this observation, a larger sample size is necessary.

To assess the beta diversity, i.e. compositional diversity between samples, the counts per library size of the three most abundant orders, Enterobacterales, Lactobacillales and Rhodospirillales, were transformed using a centered log-ratio (clr) transformation (see Fig. 9). A principal component analysis (PCA), also shown in Fig. 9, was performed on the Aitchison distance, which is the Euclidean distance for clr-transformed data between the samples [42]. The first dimension depicts the difference in Enterobacterales, while the second dimension is explained by Lactobacillales and Rhodospirillales. The cold- and hot-evolved fruit fly samples cluster together in the PCA pointing to a similarity between them. All samples collected after the egg lay ("-2") cluster on the bottom of the PCA biplot together with only one hot-evolved sample before egg lay ("-1"), which could point to a change in the microbial composition due to the egg lay.

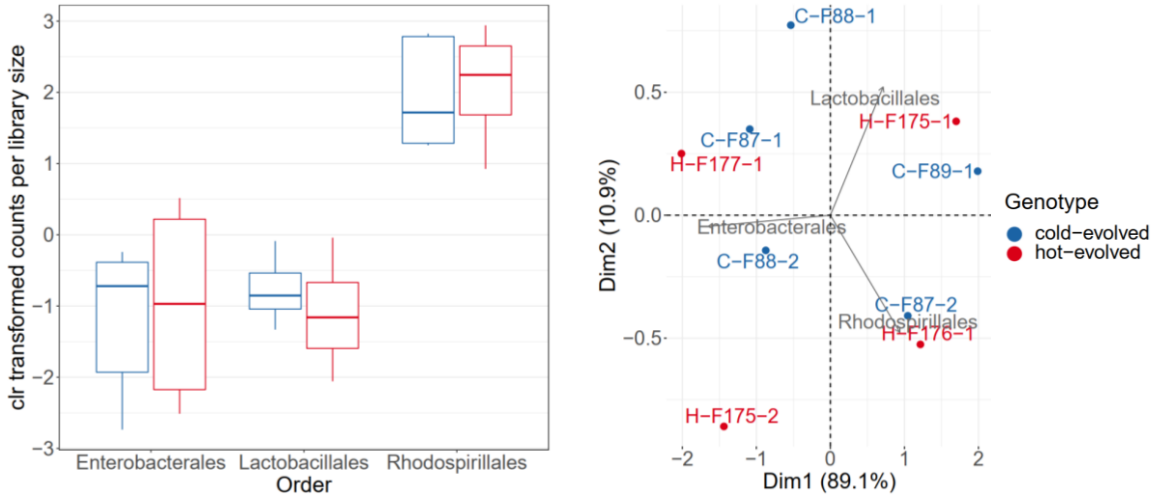

Fig. 9: Boxplot (left) and PCA biplot (right) of the clr-transformed orders: Enterobacterales, Lactobacillales and Rhodospirillales (grey in PCA biplot); All cold- (blue) and hot-evolved (red) fly samples are depicted in the PCA biplot of dimension 1 and 2.

Finally, Wilcoxon rank-sum tests were performed for each clr-transformed order separately to examine the effect of the rearing temperature of the fly populations and a Bonferroni correction was applied. No significant differences were revealed (for each order: Bonferroni corrected *p*-value = 1).

In a previous experiment, Lai et al. (submitted) generated 16S amplicon sequencing data of generation F100 from pooled flies of the hot-evolved population using Nextera Index primers. In addition, in generation F103, 16S amplicon data was obtained from five samples consisting of one male fly of the hot-evolved population collected from a common garden experiment, in which multiple replicates of the hot-evolved population were compared to each other under the same environmental conditions. During the common garden experiment, which lasted for three generations, the population size was reduced from ~1 000 flies to 300-400 flies and the time spent on the food source was increased from 1-2 days to 3-4 days. The age of the common garden flies (5 days) is comparable to the hot-evolved flies F175-F177 (4-6 days). Generations F100 and F103, which were not sequenced using the standard primers, had larger library sizes, which might have led to the higher richness found in these samples when compared to the data shown earlier (see Table 7) [40].

Table 8: All additional microbiomes sequenced by Lai et al. (submitted)

Order	F100 - pooled flies	F103 - common garden				
		1	2	3	4	5
Rickettsiales	121569	167256	153288	142090	138725	113999
Rhodospirillales	13757	6952	11062	2876	16705	11460
Lactobacillales	1005	485	1133	349	1347	778
Actinomycetales	939	206	444	117	182	245
Enterobacterales	1253	0	1	0	0	0
Pseudomonadales	16	12	265	34	65	53
Caulobacterales	0	4	32	1	175	5
Clostridiales	2	1	150	25	5	31
[Saprospirales]	3	32	38	25	23	28
Pasteurellales	0	28	46	0	1	7
Bacteroidales	0	0	16	0	0	58
Bacillales	3	10	13	20	4	19
Burkholderiales	4	6	19	14	2	2
Thermales	0	0	6	5	0	21
Streptophyta	4	0	2	0	0	12
Flavobacteriales	1	0	0	0	1	14
Gemmatales	0	0	14	0	0	0
iii1-15	0	0	0	0	14	0
Rhodobacterales	0	1	6	1	0	2
Bifidobacteriales	0	9	0	0	0	0
Solirubrobacterales	0	0	9	0	0	0
Desulfobacterales	3	0	1	1	0	0
Cytophagales	0	0	4	0	0	0
Deinococcales	0	0	0	3	0	0
Xanthomonadales	0	0	0	0	0	3
Neisseriales	0	2	0	0	0	0
Rhizobiales	0	0	0	0	2	0
Thiotrichales	0	0	0	0	1	1
Fusobacteriales	0	0	1	0	0	0
Myxococcales	0	0	1	0	0	0
Sphingomonadales	0	0	0	0	0	1
Percentage of reads assigned	99.6	100.0	99.1	100.0	99.4	98.8
Library size	139167	175035	168038	145608	158191	128338

Wilcoxon rank-sum tests revealed that generation F100 matches the generations F175-F177 in richness (p-value = 0.4) and inverse Simpson index (p-value = 0.8) (see Table 9). The richness of generation F103 is significantly higher than that of generations F175-F177 (p-value = 0.01945), but its inverse Simpson index is similar (p-value = 0.9048). Upon adding generation F100 to the data of F175-F177 a significant p-value (0.03501) for the richness can be obtained, while the inverse Simpson index remains insignificant (p-value = 0.6905). Thus, cold- and hot-evolved microbiomes should still be regarded as very similar.

Table 9: Average library size, fraction of assigned reads, richness, inverse Simpson Index and frequency of the most abundant taxa of 16S sequencing samples obtained from Lai et al. (submitted) as well as hot- and cold-evolved flies

	hot-evolved flies			cold-evolved flies
	F100	**F103**	**F175-177**	
Library size	139167	155042	18605	17749
Assigned reads (%)	99.6	99.4	99.9	99.9
Rickettsiales (%)	87.4	92.2	80.5	88.5
Without Rickettsiales				
Richness	12.0	15.8	9.25	6.4
Inverse Simpson Index	1.5	1.3	1.5	1.4
Rhodospirillales (%)	78.2	82.4	78.8	84.3
Lactobacillales (%)	5.7	7.2	3.7	6.2
Enterobacterales (%)	7.1	0.0	12.7	7.5

The beta diversity was compared by examining the orders used in the earlier analysis: Enterobacterales, Lactobacillales and Rhodospirillales. The boxplot and PCA biplot shown in Fig. 10 demonstrate that the sample of generation F100 is quite similar to generations F175-F177, unlike the common garden samples (F103).

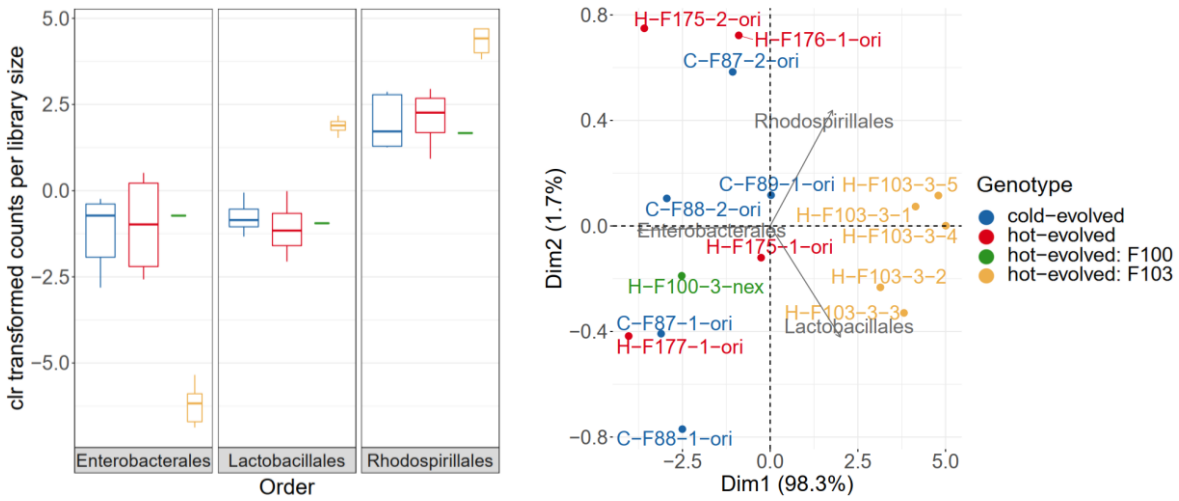

Fig. 10: Boxplot (left) and PCA biplot (right) of the clr-transformed orders: Enterobacterales, Lactobacillales and Rhodospirillales; F100 is depicted as a green dot in the boxplot.

Each order was tested using a Wilcoxon rank-sum test and all orders of generation F103 deviate significantly (p-value = 0.007937) from generations F175-F177. However, generation F100 does not differ significantly from generations F175-F177 (p-value = 1) or generation F103 (p-value = 0.3333). These results indicate that the microbiome has remained remarkably similar between generation F100 and F175-F177, which could be detected in spite of the different primer used and the big difference in library size. To statistically assess changes throughout the evolutionary line of the populations a larger sample size would be necessary.

Because generation F103 used the same primer as F100 and is of similar average library size, these circumstances can be eliminated as causes for the deviation between F103 and F175-F177. The loss of Enterobacterales could have occurred during the during the density control step, i.e. counting of the eggs, where the eggs are put in water and transferred via a pipette. Another possible explanation for the absence of Enterobacterales could be that each F103 sample consisted of only one male fly, which could imply a difference in microbial composition between sexes, that has been shown to differ by ~13 % for Proteobacteria in 3-7-day-old flies [11]. Furthermore, because the samples did not consist of pooled flies, Enterobacterales might still be present in other individuals and the sampled individual might not have come in contact with bacteria since there were less flies in the common garden population than in F100 or F175-F177. Future experiments should take this possible alteration of the microbiome into account.

3.3 Growth experiments

Colonies gathered during the culturing experiments (3.1) were used to test the hypothesis that bacteria from cold- and hot-evolved fruit fly populations adapt to their environment. Only three genera were found in the cold- and the hot-evolved populations, reared at 10-18 °C and 20-28 °C, respectively: *Acetobacter*, *Lactobacillus* and *Leuconostoc*. Sequencing of the microbiomes of these fly populations revealed that the orders these genera belong to (Rhodospirillales and Lactobacillales) are very abundant and have similar frequencies in cold- and hot-evolved microbiomes (3.2). The most abundant species of *Acetobacter* and *Lactobacillus* were chosen, *A. indonesiensis* and *L. plantarum*. The *Leuconostoc* species was always classified as *L. mesenteroides* or *L. pseudomesenteroides* and will be simplified to "*L. mesenteroides*" hereafter.

The selected strains were grown in three media (mannitol, MRS and tryptic soy) at two different temperatures, 20 °C and 28 °C. The optical density at a wavelength of 600 nm (OD_{600}) was measured at various time points following two timetables (shown in Table 12) to capture all growth phases and take into account slow- and fast-growing bacteria. The R package GrowthCurver was used to create growth curves and calculate the growth rate during the exponential phase for each strain under each condition [39]. Since many of the resulting growth curves have a large fitting error or do not cover the exponential phase (see Fig. 15), the growth rates were not used for hypothesis testing. Instead OD_{600} measurements, taken at 11 h and 12 h, were analyzed because all experiments included measurements at these two time points. Both analyses returned very similar results, thus only results for the 11 h measurements are shown. Boxplots of the 11 h measurements can be found in Fig. 11 and 12 h measurements in Fig. 14 in the Appendix.

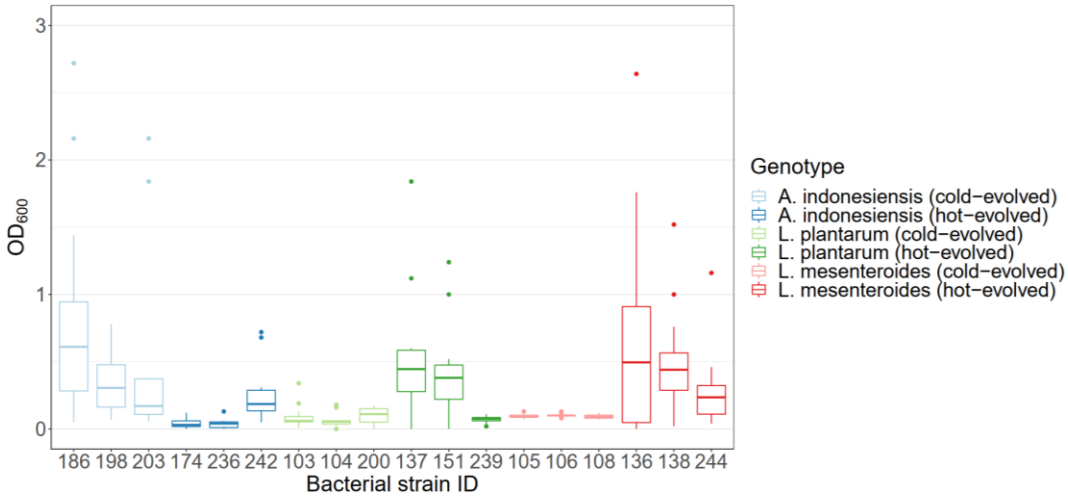

Fig. 11: Boxplots of 11 h measurement points combining all media and culturing temperatures from both timetables

For each species a linear mixed effects model was used to analyze the significance of the rearing temperature of the flies, the culturing temperature of the bacteria and the medium. The boxplots of these three parameters can be found in Fig. 12 and the results of the model are shown in Table 10. Among all species MRS was a significantly worse medium than mannitol and tryptic soy. Although the highest OD_{600} measurements for all species were obtained in tryptic soy, the measurements taken in mannitol and tryptic soy are not significantly different. The rearing temperature of the flies is only significant before Bonferroni correction, while the culturing temperature is significant even after multiple testing correction. Both cold- and hot-evolved *A. indonesiensis* and *L. plantarum* grew better at 28 °C. A significant interaction between rearing and culturing temperature can only be found in *L. mesenteroides*: For this species the hot-evolved bacteria grew the best at 28 °C, while the cold-evolved bacteria grew faster at 20 °C. In the model including this interaction the culturing temperature is insignificant, but in a model without the interaction it would be significant.

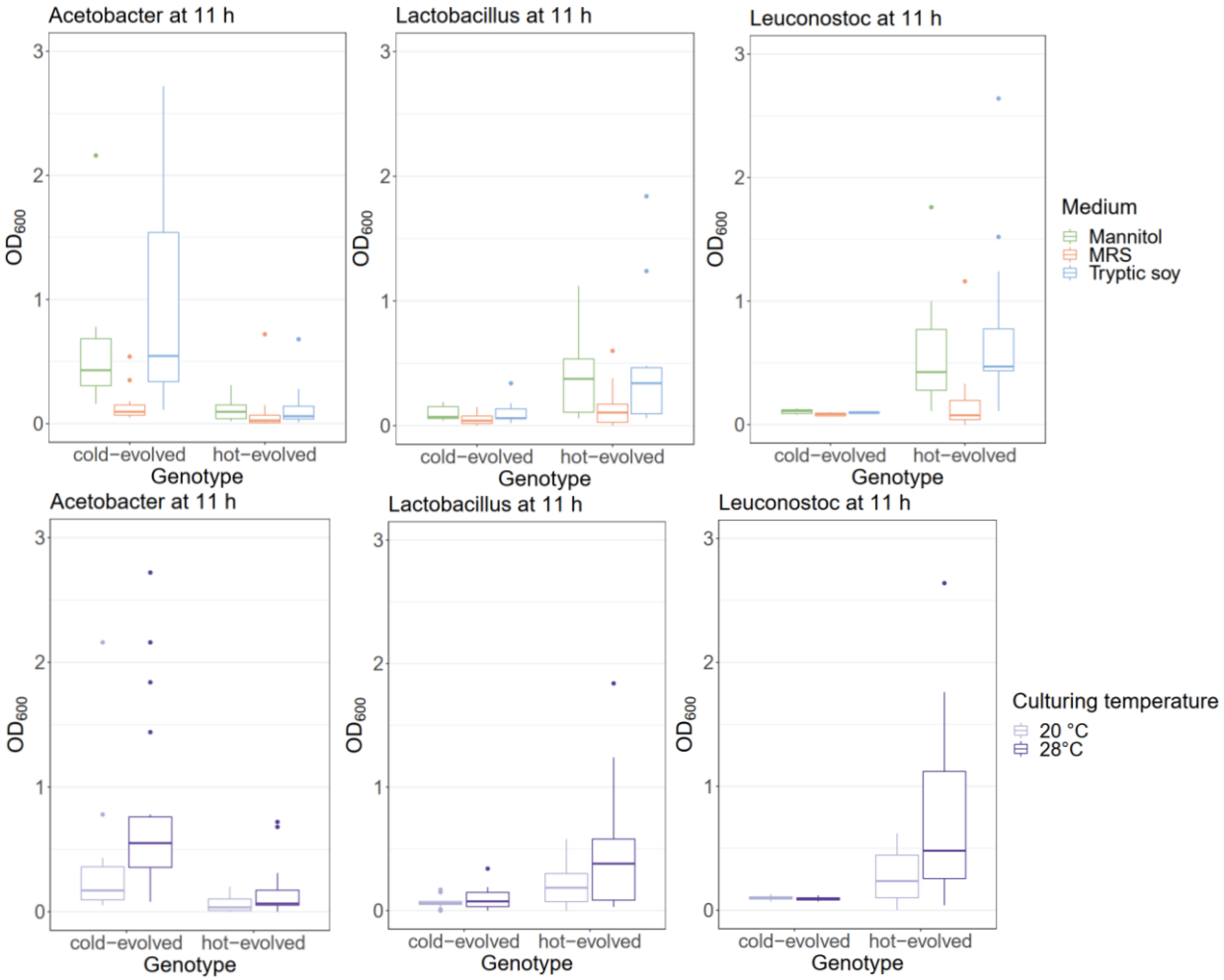

Fig. 12: Boxplots of the media (top) and culturing temperature (bottom) used in the growth experiments of *Acetobacter* (left), *Lactobacillus* (middle) and *Leuconostoc* (right) at 11 h

Table 10: Compiled list of results from all linear mixed effects models: The column "Medium" states which medium is used as the starting point of the comparison.

Species	Fixed effect	Estimate	Medium	*p*-value	Bonferroni corrected *p*-value
Acetobacter indonesiensis	CulturingTemp28	0.451389321		2.14E-10	6.43E-10
	RearingTemp18-28	-0.674685109		0.024136194	0.072408583
	MediumMRS	-0.476477258	Mannitol	1.47E-08	4.42E-08
	MediumTryptic soy	0.02218467	Mannitol	0.764611538	1
	MediumTryptic soy	0.498661927	MRS	4.35E-09	1.30E-08
Lactobacillus plantarum	CulturingTemp28	0.238474629		0.005221693	0.015665079
	RearingTemp18-28	0.503660474		3.91E-02	1.17E-01
	MediumMRS	-0.401432412	Mannitol	0.000179502	0.000538506
	MediumTryptic soy	0.009401236	Mannitol	9.26E-01	1.00E+00
	MediumTryptic soy	0.410833648	MRS	0.000130792	0.000392375
Leuconostoc mesenteroides	CulturingTemp28	-0.019757751		0.836262967	1
	RearingTemp18-28	0.203111125		0.036389335	0.109168005
	RearingTemp18-28:CulturingTemp28	0.468716244		0.000856713	0.002570139
	MediumMRS	-0.385509734	Mannitol	1.34E-05	4.03E-05
	MediumTryptic soy	0.025921239	Mannitol	0.754253676	1
	MediumTryptic soy	0.411430973	MRS	4.09E-06	1.23E-05

4. Discussion

In this thesis, multiple experiments were performed to study the microbiome of *D. simulans*. During the culturing experiments questions about how the microbial composition is affected by oxygen and short- as well as long-term changes in temperature were addressed. These long-term changes in temperature were also examined by 16S rRNA sequencing of the microbiome of cold- and hot-evolved fly populations. Furthermore, growth experiments on bacterial strains collected from these populations were used to find out more about the effect of temperature on the growth of the bacterial strains.

4.1 Cultured microbiomes of *D. simulans* populations

Cultures of fruit fly populations grown at different temperatures showed that not all bacteria can grow at 37 °C. Thus, to obtain a more accurate representation of the culturable microbiome the bacteria should be grown at the rearing temperature of their fly hosts. Comparing cultures reared at the same temperature revealed a large variation in microbial composition, which is common among laboratory populations [10][12]. The an- and aerobic microbiomes of all samples except for one did not differ much. Since the culturing experiments were made without replicates the variation between samples was not taken into account and they should be repeated with replicates to establish more trustworthy depictions of the culturable microbiomes.

The colonies of these experiments that were classified and showed clear phenotypic traits can be preliminarily identified in future studies. Furthermore, contamination will be more easily detected if unusual phenotypic traits, e.g. the orange color of *Dermacoccus*, are found on plates.

Since the cold-evolved *Acetobacter indonesiensis* colonies had varied growth on the three media and some exhibited different colors, 15 of the collected strains will have their whole genome sequenced. This should reveal if their phenotype is very plastic or if changes in their DNA sequence are responsible for these observations. Besides these strains, five bacteria with heterozygous regions in their 16S rRNA gene or unclear classification will also be sequenced.

4.2 16S rRNA amplicon sequencing

Primer comparison

The counts obtained by using different primers for 16S amplicon sequencing were compared and sequence alignments revealed that most counts, that were lower than expected (binomial distribution probability), were not caused by mutations in the 16S rRNA gene sequence of the bacteria. This could point to a problem during the amplification process. Furthermore, the 670 bp data set might not be a good reference for the microbial composition, because 670 bp's inability to amplify the most abundant orders might have resulted in an overrepresentation of less frequent orders. This overrepresentation might be advantageous for future studies of less prominent bacteria. Since the most abundant orders are of particular interest, they should be examined using primers that can detect them. A more accurate number of counts might be obtained by amplifying the 16S rRNA gene using a different primer that is able to amplify all bacteria equally well. A starting point could be the primers from Fuks et al. (2018).

Cold- and hot-evolved microbiomes

Although the hot-evolved population had a higher number of total orders that was almost significant (without adding generation F100 of the hot-evolved population to the data set), their effective number of orders was not significantly higher than that of the cold-evolved flies. Thus, the cold- and hot-evolved microbiome consist of a similar number of bacteria. The overall low number of effective orders indicates that the gut microbiome is made up mostly of one order, namely Rhodospirillales. The composition of the three most abundant orders did not vary significantly between cold- and hot-evolved flies, which is remarkable considering that laboratory populations of *Drosophila* have been shown to contain different microbial communities [10][12].

Moghadam et al. (2018) showed changes in microbial composition of the medium and the gut of *D. melanogaster* flies after changing the rearing temperature from 25 °C to either 13 °C, 23 °C or 31 °C. At the lowest temperature *Leuconostoc* (order Lactobacillales) was the most abundant taxon in the food source and the second most abundant in the fly gut after the endosymbiont *Wolbachia*. Upon raising the temperature, the amount of *Leuconostoc* and *Wolbachia* decreased. In contrast, *Acetobacter* (order Rhodospirillales), which made up only a small percentage of the microbial composition at 13 °C, became the most abundant taxon at higher temperatures except for one condition: 31 °C in the medium. *Lactobacillus* (order Lactobacillales) was not very prominent and no taxa of the order Enterobacterales were very abundant. The changes in microbial composition found in this study do not match the similarity between the microbiomes of the cold- and hot-evolved populations. However, the differences between these studies might stem from Moghadam et al. (2018) studying a short-term temperature change whereas the two evolved populations are an example for a long-term change in temperature. A similar increase of Proteobacteria (Rhodospirillales, Enterobacterales) has been associated with rising temperatures in wood lice (*Porcellio scaber*) and *Caenorhabditis elegans* [6]. [27]

4.3 Growth experiments

The bacteria grew significantly better at 28 °C than at 20 °C. Only the cold-evolved *L. mesenteroides* grew worse at 28 °C. Although the growth difference was small, this interaction between the rearing temperature of the fruit flies and the culturing temperature was found to be significant. *L. mesenteroides* should be cultured once more and measured at later time points to examine if this effect persists at higher growth and if the difference between the cold- and hot-evolved strains increases or stays at the same level.

Interestingly, *A. indonesiensis* originating from cold-evolved fly populations grew a lot better at both culturing temperatures than the same species collected from hot-evolved fly populations. In contrast to this, both *L. plantarum* and *L. mesenteroides* presented the exact opposite growth behavior. Since both *Acetobacter* and *Lactobacillus* have been shown to proliferate in the gut, the microbiome composition could be influenced by competition either inside the gut or in the food source [18].

If an early abundance of Lactobacillales can be viewed as the original state, then the fast-growing *Acetobacter* of the cold-evolved population could affect the initial microbiome of the larvae and lead to an early microbiome consisting of mostly *Acetobacter*. An experiment using *D. melanogaster* populations kept in outdoor mesocosms with a food source enhanced with either *L. brevis* or *A. tropicalis* for >40 days might shine a light on why having a relative abundance of these genera is important. The populations treated with *A. tropicalis* had a higher mass than the populations treated with *L. brevis*, but those treated with *L. brevis* had a larger population size. The higher mass might be related to *A. tropicalis*' ability to achieve faster development times than *L. brevis* [43]. Another study revealed that flies monoassociated with *Acetobacter* species lead to earlier fecundity than *Lactobacillus* species but the number of offspring and the fitness is similar between both conditions [44]. In context with this study's findings of increased growth of *A. indonesiensis* collected from cold-evolved hosts, an early increase in *Acetobacter* might improve survivability of the larvae in the cold environment, that is later traded for a more balanced microbiome including *Lactobacillus* to improve nourishment (triglyceride reduction) [45] and reproduction. [21]

Furthermore*, Lactobacillus* has been shown to protect its host from pathogens like *Serratia marcescens* and *Pseudomonas aeruginosa* [17]. *Acetobacter* might also lead to such an advantage for its host that is prioritized in cold-evolved flies. Whether *Leuconostoc* also benefits its host besides also speeding up development should be investigated as well [43].

An important fact to keep in mind is that while the strains were assigned to the same species there might still be high functional diversity within each species. Significant differences in copy number variation have been shown in human gut microbiome strains, especially regarding genes affecting transport and signaling processes [46]. The same study also found that most microbiomes consisted of multiple strains of each species, which has also been shown in honey bees [47]. Arnold et al. (2018) presented strains of *L. rhamnosus* that exhibit varying stress resistances [48]. Rather than being an example of strains adapting to temperature the growth patterns might show the potential plasticity of the microbiome by favoring strains that suit the environment. Toxin-antitoxin gene markers have been used to identify strains of *L. plantarum* and other species in human gut microbiomes and might be applicable to *Drosophila* samples to find out if the same strains can be found in both populations [49][50]. All strains used in the growth experiments will be whole genome sequenced to find out more about their functional differences.

5. Conclusion

In this study a first look was taken at the microbiome of various *D. simulans* strains of the IPG and protocols were set up that will be valuable for future experiments. Three bacteria were shown to have adapted to their host's environment and will certainly be a topic of further research. The similarity in the microbiome and the gut between *Drosophila* and other animals, particularly insects, should make the knowledge gained during this study useful in a wide array of sectors, e.g. conservation, agriculture, livestock and even biomedicine.

6. Material & Methods

6.1 Establishment of the culturing protocol

The following protocol for culturing, identifying and storing bacteria isolated from fruit fly homogenate was created after preliminary tests were performed, that can be found in the *Appendix*. All experiments were performed according to this protocol unless stated differently.

Media overview and plating

De Man, Rogosa and Sharpe (MRS) as well as tryptic soy agar were bought in powder form, while mannitol agar needed to be prepared (see recipe in *Appendix*). MRS medium was developed to grow *Lactobacilli* [51] and mannitol medium favors *Acetobacter* [52]. In contrast to the other two media, tryptic soy agar is non-selective [53]. After preparation, all media were stored at 5 °C before plates were poured, which were also stored at this temperature after cooling.

Adult flies were put in three Eppendorf tubes filled with 100 individuals each. 100 µL autoclaved 1X Phosphate Buffered Saline (PBS) (see *Appendix*) were added to each tube and the flies were crushed using a pestle. After shortly spinning the samples, 1 µL homogenate was taken from each tube and put in a new tube filled with 147 µL 1X PBS. After mixing, 10 µL of this homogenate were diluted in 90 µL 1X PBS (1:10). Then another dilution (1:100) was made by adding 10 µL of the first dilution to 90 µL 1X PBS.

All plating of bacteria was done close to a flame for sterility. First the homogenate was placed on the plate, then a cell spreader was sterilized by rinsing it with 96 % denatured EtOH and setting it on fire. To cool it off, the cell spreader was pressed on the surface of the plate next to the liquid. Streaking was performed while slowly turning the plate until the fluid had dried out. Plates for aerobic bacteria were put directly into the incubator. To recreate the anoxic environment of the midgut for anaerobic species, the plates were put in a tightly shut glass container and the amount of oxygen was reduced by lighting a small candle before putting it in the incubator as well.

Colony streak test

After two days ~50 colonies were chosen at random from each medium, whilst incorporating every unique phenotype, and transferred to 3 plates, one of each medium, using autoclaved toothpicks. The original medium was always plated last. MRS was plated first, unless it was the original medium, because colonies grew slower on it (see *Technical Test*). Upon completion the plates were cultivated as described earlier.

The resulting colony streak test plates were analyzed for each medium separately, and colonies were put into four categories depending on their growth:

- no growth
- little growth
- good growth
- exceptional growth

Characteristics like color and discernible surface structures were also noted. Out of all colonies around 30 were further analyzed.

PCR, sequencing and identification

A small region of the 16S rRNA gene was amplified via PCR using the "670 bp" primer set based on Fuks et al. (2018) by adding a small amount of the colony to the reaction mix (see *Appendix*). To create more PCR product, the reaction mix was altered to achieve a total volume of 50 µL instead of 20 µL. If there was only very little material available or the PCR did not work, a liquid culture was set up and used for the PCR instead. Because some colonies did not produce PCR products, although the liquid cultures grew well, another forward primer was designed based on the primers used for testing axenic flies. This "16S long" primer set was able to amplify the colonies, which were determined to belong to the genus *Acetobacter*.

All resulting fragments were purified using the QIAquick® PCR Purification Kit and measured using the Qubit DNA HS kit. If the 200 ng needed for Sanger sequencing by LGC Genomics GmbH (Ready 2 Run program) were not achieved, the PCR was repeated. The acquired sequences were checked for inconsistencies using the program CodonCode Aligner [54] and were assigned using blastn [37].

Liquid cultures and freezing

Colonies were cultured in eprouvettes using their best growing medium and shaken at the same temperature used in the experiment until they were turbid, after which they were stored at 5 °C. Before fluid was taken out of the culture a pipette was used for mixing. After determining the identity of the samples, 0.5 mL of the liquid cultures were mixed with 0.5 mL 50 % autoclaved glycerol and frozen at -80 °C after vortexing and very shortly spinning down the mixture.

Technical test

An experiment was designed to test, if there were any differences between the growth of colonies applied first, second or last during the colony streak test. An *E. faecalis* colony from the frozen glycerol stocks, that grew better on mannitol than it did on tryptic soy agar, was chosen for testing. First a small amount of frozen stock was streaked on a mannitol plate with an inoculation loop in 3 areas and put at 18-28 °C overnight under oxic conditions. One colony from this plate was then streaked on 2 plates of mannitol agar, followed by 2 plates of tryptic soy agar and finally 2 plates of MRS agar using just one toothpick. This was repeated 10 times. Additionally, 3 colonies were applied the other way around and 5 series of streaks were made using inoculation loops in the order described first. All plates were put at 18-28 °C for two days.

Out of the streaks repeated 10 times 9 looked similar. Although the original colony grew better on mannitol than on tryptic soy, during this experiment the colonies grew to a similar size. The 3 colonies applied in the reverse order resembled the ones applied first to last. All colonies streaked using inoculation loops grew similarly. Since no colonies could be seen on the MRS agar plates, these plates were put on 18-28 °C for four more days. After the colonies grew to a bigger size, these two plates looked the same as the earlier ones.

6.2 16S amplicon sequencing

Using the newly established protocol cultures were grown from two populations of *D. simulans* from Florida, that were reared at temperatures between 18 °C and 28 °C or between 10 °C and 20 °C, switching every 12 hours, for 175 and 87 generations, respectively [33]. For the first sample listed in Table 11 a second culture was made, because the first one appeared to grow unusually slowly compared to earlier experiments. Only anaerobic cultures were made and about 30 colonies were chosen for the colony streak test from all media.

Table 11: Cold- and hot-evolved fruit fly populations used for Illumina sequencing, culture and growth experiments

Generation	After egg lay	T (°C)	Age (days)	Illumina sequencing	Culture	Colony streak test	Growth experiment
				No. of flies	Time (days)	Time (days)	
87	no	20/10	4-8	300	6, 4	4	yes
87	yes	20/10	10-14	300	2-11	3-4	yes
88	no	20/10	6-8	300	5-16	5	no
88	yes	20/10	13-15	200	5-10		
89	no	20/10	8	300			
175	no	28/18	4-6	300	3-8	5	yes
175	yes	28/18	7-9	300			
176	no	28/18	4-6	300	2	2	yes
177	no	28/18	8	300			

Cold- and hot-evolved microbiomes

In addition to culturing bacteria from the cold- and hot-evolved flies, their microbiome was analyzed by sequencing whole flies. For all but one sample (see Table 11) 300 flies were put into an Eppendorf tube filled with 600 µL of HOM buffer and crushed using a pestle. DNA-extraction was performed according to the protocol described under *Appendix*. Illumina 16S amplicon libraries were prepared by Viola Nolte, Dipl.-Biol., as in Lai et al. (submitted). For the comparison of cold- and hot-evolved flies IPG's standard primer set was used, which is the recommended by Illumina for 16S amplicon sequencing [55]. Additional samples were amplified using two other primers, "670 bp" and "16S long", that were also used to identify colonies of these populations.

After demultiplexing, the interleaved reads were trimmed to a minimum read length of 76 bp and a quality threshold of 18, while ignoring 5'-ends. Two interleaved fastq-files per sample were used in a metagenomic analysis via the program Kraken 2 [56] and the database GreenGenes [57], customized to 125 bp k-mers to better fit the average read length of 123 bp. The classifications were entered into the program Bracken to estimate the total reads [58]. Bracken re-distributes reads that could only be identified at a certain level by Kraken 2 to lower levels according to probabilities that are calculated based on Bayes' theorem. The taxonomic level of order was chosen, because most reads could be assigned at this level on average 98.5 % and 99.9 % for two of the primers used, while the last primer set, 670 bp, only resulted in an average of 63.7 %.

All subsequent analyses were performed using the R 3.6.3 statistical environment (R Development Core Team, 2006). A list of all packages that were used can be found under *Appendix*.

The parasitic endosymbiont *Wolbachia*, order Rickettsiales, was removed from all samples before calculating the counts per library size. To assess the alpha diversity, which is the diversity between samples, the package "vegan" (see *Appendix*) was used to obtain the richness and the inverse Simson index, i.e. effective number of orders. Wilcoxon rank-sum tests were used to compare the richness and inverse Simpson index of the cold- and hot-evolved samples. For the beta diversity, i.e. the compositional diversity of the samples, the three most abundant orders found in all samples were selected and a centered log-ratio transformation was performed. Using these transformed data, a PCA biplot was made using the package "factoextra" (see *Appendix*) and Wilcoxon rank-sum tests were performed on each order separately to compare the cold- and hot-evolved fruit flies. [59][41]

Pooled sequencing data of generation F100 of the hot-evolved population and five samples consisting of one male fly of generation F103 collected after a common garden experiment were added to the data set of the cold- and hot-evolved flies. Following the pipeline described earlier, alpha and beta diversity were studied. Additionally, comparisons between the hot-evolved generations 175-177, 100 and 103 were tested using Wilcoxon rank-sum tests.

Primer comparison

This data set consists of 3 samples, generation 87 of the cold-evolved population before and after the egg lay and generation 175 of the hot-evolved population before the egg lay, that were amplified using three different primer pairs: IPG's standard, 670 bp and 16S long. Since many orders were not amplified by one of the three primers or seemed much lower in the samples of one particular primer, sequence alignments using blastn were made between the primers and the 16S rRNA gene of each species [37]. The species were chosen after looking up the genera identified by Kraken 2 and Bracken. Sequences of the species were obtained from the NCBI database [60] and the names were checked using the LPSN [61]. Rickettsiales and Rhodospirillales were examined and can be found in Table 16 under *Appendix*.

All other orders were ranked according to how many of the 670 bp samples they were found in. Using the frequency of the orders found in at least two samples, binomial distribution probabilities were calculated for the counts of the other two primers. Library sizes without Rickettsiales and Rhodospirillales were used as the number of trials, because these highly abundant orders would skew the results. Table 17 featuring these probabilities as well as sequence alignments can be found under *Appendix*. Two more tables were made using the same process for 16S long and standard samples excluding orders that already appeared in previous tables (see Table 18).

6.3 Growth experiments

During the growth experiments cultures were grown at either 28 °C in eprouvettes on a heating shaker set to 220 rpm, or 20 °C in falcon tubes on a rocking platform shaker at maximum speed set up in an incubator. Although the latter moved slower the horizontal shaking allowed for more movement inside each tube. Samples were measured on an Ultrospec 10 spectrophotometer from Biochrom US. All semi-micro cuvettes were reused up to two times after carefully being washed with distilled water and drying completely. Before measuring, an uncultured medium was set as reference.

Preliminary experiments pointed to an exponential phase between 8-24 hours. For each bacterial strain two separate experiments were measured to obtain a full picture of the growth curve, from lag to stationary phase, while also taking into account fast and slow growing cultures. For each day and night tests, named after the starting time, 11 measurements were taken (see Table 12), one less than the maximum possible for strains that did not grow at all in a specific medium.

Table 12: Measurement points (hours) in the growth experiments

Day regime	1	3	5	7	9	11	12	/
Night regime	/	/	/	/	/	11	12	13
Day regime	/	/	/	24	25	27	/	*>3 days later*
Night regime	15	18	20	/	/	/	44	*>3 days later*

Because of time restraints and lack of space only one replicate of each culture could be measured. To confirm that this would be reproducible a day regime experiment was run with four replicates of the same *L. plantarum* strain and resulted in very similar growth curves (see plots under *Appendix*).

Growth rates were calculated using the R package GrowthCurver and lead to bad fits, especially for samples with only low OD_{600} values [39]. Instead of using these growth rates, analyses were performed on 11 h and 12 h measurements separately. Although growth data for *Dermacoccus nishinomiyaensis* were collected, they were removed from the analysis, because this bacterium was not found in the sequencing data and is suspected to originate from contamination. Thus, only measurements of *A. indonesiensis*, *L. plantarum* and *L. mesenteroides* were analyzed.

The 11 h measurement values of the three species were analyzed separately using the following parameters: strain ID, rearing temperature of the fruit flies, culturing temperature of the bacteria, medium and testing scheme. A linear mixed-effects model was fitted to the 10th logarithm of the OD_{600} measurements of. Normalcy and homogeneity plots can be found in Fig. 16. The strain ID was used as a random effect, while the testing scheme was left out, because it only has two levels [62]. The fixed effects consisted of the rearing temperature, the culturing temperature and the medium. All possible interactions between these factors were tested, but all except one were insignificant. Thus, only for *L. mesenteroides* an interaction between the two temperatures was used. [41]

The same analysis on the 12 h measurement data returned very similar results.

7. Acknowledgements

I want to thank Dr. Christian Schlötterer for his great advice and enthusiasm for this study. Regarding the practical side of this thesis I would like to thank Viola Nolte, Dipl.-Biol., for making me feel very welcome, guiding me throughout my work and helping me solve all problems that arose. Furthermore, I want to thank Emmanouil Lyrakis, M.Sc., for deepening my knowledge of regression models and looking over my analysis as well as this report. Additionally, I want to thank Rupert Mazzucco, Dr.rer.nat., for taking the time to explain the programs Kraken and Bracken to me, and my good friend Julia for creating the graphic that shows my approach for collecting bacterial strains. Finally, many thanks to my family and friends for all their support during my academic education.

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9. Appendix

9.1 Zusammenfassung

In vielen vor kurzem erschienenen Studien über das Mikrobiom wurde *Drosophila* als Modellorganismus ausgewählt, da das Mikrobiom der Fruchtfliege im Vergleich zum Menschen weniger komplex ist. Über den Einfluss der Mikroorganismen auf das Verhalten und die Wachstumsrate ihres Wirts, aber auch über den Einfluss des Wirts auf die mikrobielle Zusammensetzung ist bereits viel bekannt. Das Ziel dieser Studie ist es, Unterschiede in der Komposition zwischen zwei evolvierten Populationen, die unter spezifischen Umweltbedingungen gehalten wurden, zu identifizieren und an Hand der Wachstumsraten verschiedener Bakterienspezies die funktionelle Diversität zwischen den Stämmen mit unterschiedlichen evolutionären Hintergründen zu zeigen.

Zwei *Drosophila*-Populationen aus Florida, die entweder bei 10-20 °C für 87 Generationen oder bei 18-28 °C für 175 Generationen gehalten wurden, wurden mit Hilfe der folgenden Methoden untersucht: Zuerst wurde das Mikrobiom auf Agarplatten kultiviert und Kolonien mit differenzierbaren Phänotypen identifiziert und für spätere Analysen eingelagert. Danach wurde das gesamte Mikrobiom der heiß- und kalt-evolvierten Populationen sequenziert und analysiert.

Die heiß-evolvierte Population weist zwar eine höhere Anzahl an unterschiedlichen Bakterien auf, die effektive Anzahl an Ordnungen der zwei Populationen ist jedoch sehr ähnlich. Die Abundanz der drei häufigsten Taxa ist ebenfalls vergleichbar. Obwohl sich die zwei Populationen auch in Bezug auf die Ordnungen, aus denen ihr Mikrobiom besteht, sehr ähneln, konnte bei Wachstumsversuchen gezeigt werden, dass sich die Bakterienstämme der drei häufigsten Spezies unterschiedlich verhalten.

Zwei Spezies, die aus dem Mikrobiom der heiß-evolvierten Fliegen stammen, wuchsen signifikant schneller als ihre Äquivalenten aus den kalt-evolvierten Fliegen, wohingegen die letzte Spezies ein umgekehrtes Wachstumsverhalten aufwies. Diese Wachstumsmuster könnten entweder durch die Anpassung der Bakterien auf die Umgebung des Wirts entstanden sein oder weisen auf die Diversität von Stämmen, die der gleichen Bakterienspezies zugeordnet werden, hin. Da die relative Abundanz zwischen den zwei Haltungsbedingungen vergleichbar war, könnte dieses Wachstumsmuster auf Unterschiede beim erstmaligen Aufbau des Mikrobioms hinweisen. Bei zukünftigen Studien über das Mikrobiom und im Speziellen über dessen Aufbau sollte beachtet werden, dass Stämme, die der selben Spezies angehören, in verschiedenen Wirtspopulationen nicht die gleiche Wachstumsrate haben, und sich wahrscheinlich in einigen physiologischen Prozessen unterscheiden.

9.2 Abbreviations

ANOVA	analysis of variance
clr	centered log-ratio
EtOH	ethanol
IPG	Institute of Population Genetics
MRS	De Man, Rogosa and Sharpe medium
OD_{600}	optical density at an absorbance of 600 nm
OTU	operational taxonomic unit
PBS	phosphate-buffered saline
PCA	principal component analysis
T	temperature in Celsius
Tm	melting temperature in Celsius

9.3 Media

De Man, Rogosa and Sharpe (MRS) medium, Roth: X924.1, X925.1

- agar 12.4 g for 200 mL (prepared with distilled water)
- broth 10.4 g for 200 mL (prepared with distilled water)

MRS media varied in color, although they were made from a formula.

Tryptic soy medium, Roth: CP70.1, X938.1

- agar 8 g for 200 mL (prepared with distilled water)
- broth 6 g for 200 mL (prepared with distilled water)

Mannitol agar and broth mixed according to protocols by HiMedia Laboratories [52] [63]

- Peptic digest of animal tissue, Roth: 2366.1
- Mannitol, Roth: 4175.1

The stated pH of 7.4±0.2 at 25 °C was not reached. The broth had a pH of 6.5 at RT, while the agar had between 6-7.
The mannitol plates used in the first culturing experiments was made using Danish agar instead of Kobe agar.

9.4 1X Phosphate Buffered Saline (PBS buffer)

1. Dissolve the following in 800 mL distilled H2O
    - 8 g of NaCL
    - 0.2 g of KCl
    - 1.44 g of Na2HPO4
    - 0.24 g of KH2PO4
2. Adjust pH to 7.4 with HCl
3. Adjust volume to 1 L with additional distilled H2O
4. Sterilize by autoclaving

Resulting in:
137 mM NaCl
2.7 mM KCl
10 mM Na2HPO4
2 mM KH2PO4

9.5 Sugar-agar plates for egg laying

1. For circa 20 plates use
    - 8 g sugar
    - 8 g agar
2. Fill up to 200 mL with desalted H_2O
3. Autoclave and pour plates

9.6 16S rRNA primer sets

All primers were ordered from Sigma-Aldrich Handels Gmbh.

<u>16S long primers</u>

16S long fw	5′-AGGATTAGATACCCTGGT-3′	Position: 752	Tm: 52.1 °C
16S long rv	5′-CCCGGGAACGTATTCACC-3′	Position: 1336	Tm: 65.1 °C
	3′-GGTGAATACGTTCCCGGG-5′		

<u>670 bp primers</u>

670 bp fw	5′-GTGTAGCGGTGAAATGCG-3′	Position: 650	Tm: 63.0 °C

reverse primer: see 16S long rv

<u>Standard primers</u>

forward	5′-CCTACGGGNGGCWGCAG-3′	Tm: 65 °C
reverse	5′-GACTACHVGGGTATCTAATCC-3′	

<u>Primers used by Kietz et al.</u>

forward	5′-AGAGTTTGATCCTGGCTCAG-3′	Position: 8F	Tm: 61 °C
reverse	5′-GWATTACCGCGGCKGCTG-3′	Position: 1336	Tm: 64.6°C

9.7 DNA extraction and PCR protocol

Based on "Salt Extraction of genomic DNA" (Emmanouil Lyrakis, M.Sc.), which is adapted from: Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 16, 1215.

DNA extraction

- put 200 µL HOM Buffer (with Protease K added) into an Eppendorf tube
- add flies and crunch with beads and incubate at 58 °C o/n (at least 4 – 6 h)

- add 100 µL (1/2 volume) 4.5 M NaCl and mix (vortex + spin down 4 s)
- add 225 µL (0.75 volumes) of chloroform and mix for 10 min on rotator
- centrifuge for 10 min at maximum speed
- transfer upper phase into new tube (≈ 200 µL)
  Attention: avoid the interphase!
- precipitate with 1 Vol. (200 µL) 100 % Isopropanol, shake thoroughly or vortex shortly, then immediately centrifuge for 10 min at max. speed (4 °C)
- decant the isopropanol – **Attention: the pellet likes to swim away!**
- wash with 0.5 mL 70 % EtOH
- incubate for 15 min at RT
- centrifuge for 10 min at max. speed (4 °C)
- decant alcohol (*visual control that the pellet stays where it should!*) + pipette out remaining fluid
- dry at RT (for 25 min – o/n with paper towel over it), if not dry: 5 min speed vacuum
- dissolve in 30 µL H_2O (vortex + 5 min rest at RT, then vortex + spin down 4 s)

Treatment with RNaseA (optional)

If you need RNA-free DNA, do the following:
- add 2.0 µL of the RNAseA (1.0 µg/mL) to the homogenate after the 58°C incubation
- incubate for 15-30 min. at room temperature, then continue with addition of 4.5M NaCl

Alternatively:
There is the possibility to do the RNase A treatment at the end of the DNA extraction:
- add 1µL of the RNAseA (1.0 µg/mL) to your DNA extract
- incubate for 10 min. at room temperature or 37°C

Run the samples in a 1.0 % agarose gel

Small electrophoresis chamber: 40 mL

0.4 g	agarose for genomic DNA (for PCR products use 0.6 g)
40 mL	1X TAE
1 µL	ethidium bromide

Load 2 µL of Gene Ruler 100 bp Plus (1:20 dilution in 1X Loading dye) in one well and 1µL of your DNA mixed with 1µL 1X Loading dye in the other wells.

Gel electrophoresis settings:
genomic DNA: 120 V, 15 min, 700 current
PCR product: 140 V, 15 min, 700 current

PCR with Fire Taq

per sample in a final PCR volume of 20 µL with 1.0 µL template DNA:

11.92 µL Milli-QH_2O (use H_2O to adjust to final volume of 20 µL)
2.0 µL 10X Fire Taq buffer B (doesn't contain $MgCl_2$)
2.0 µL 25 mM $MgCl_2$ (final concentration of 2.5 mM is strongly recommended)
0.5 µL 20 µM forward primer
0.5 µL 20 µM reverse primer
2.0 µL 2 mM dNTPs
0.08 µL Fire Taq (5 U/µL – final concentration 0.4 U) (on ice)

Cycling conditions:

Cycle step	Temp./°C	Time	Cycles
Initial denaturation	95	3-5 min	1
Denaturation	95	30-60 s	
Annealing	*	30-60 s	*
Elongation	72	1 min/Kb	
Final Elongation	72	7 min	1

After that 20 °C or RT is fine. Duration: ~ 1 h

* *wsp* + Lv: 55 °C, 32 cycles
Kietz: 52 °C, 20 cycles
16S long: 55 °C, 22 cycles
670 bp: 60 °C, 30 cycles

Run the samples in a 1.0 % agarose gel.

see above

Reagents required:

1) Homogenization Buffer

(final concentration: 160 mM Sucrose, 80 mM EDTA (pH 8.0), 100mM Tris (pH 8.0), 0.5 % SDS, 0.10 mg/mL Protease K)

Preparation of 250 mL:

13.69 g Sucrose
40 mL EDTA – Stock solution: 0.5 M, pH 8
25 mL Tris-Cl – Stock solution: 1 M, pH 8
12.5 mL SDS – Stock solution: 10 %

Before using: add 50 µL Protease K (10 mg/mL) to 5 mL HOM Buffer
Note: HOM Buffer with Protease K added has to be stored at -20 °C (w/o Prot K at RT)

2) 4.5 M NaCl:

Preparation of 100 mL:

26.3 g NaCl

3) Chloroform
4) 100 % Isopropanol p.a.
5) 70 % Ethanol, made from Ethanol absolute, p.a.

9.8 Software, databases and R packages

Kraken 2.0.8
Bracken 2.5.0
GreenGenes version 2019-04-18
RStudio 1.2.5
Microsoft Office 365

R packages

car
John Fox and Sanford Weisberg (2019). An {R} Companion to Applied Regression, Third Edition. Thousand Oaks CA: Sage. URL: https://socialsciences.mcmaster.ca/jfox/Books/Companion/

compositions
K. Gerald van den Boogaart, Raimon Tolosana-Delgado and Matevz Bren (2020). compositions: Compositional Data Analysis. R package version 1.40-5.

data.table
Matt Dowle and Arun Srinivasan (2019). data.table: Extension of `data.frame`. R package version 1.12.8.

dplyr
Hadley Wickham, Romain François, Lionel Henry and Kirill Müller (2020). dplyr: A Grammar of Data Manipulation. R package version 0.8.5.

factoextra
Alboukadel Kassambara and Fabian Mundt (2020). factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.7.

ggplot2
H. Wickham. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.

gridExtra
Baptiste Auguie (2017). gridExtra: Miscellaneous Functions for "Grid" Graphics.

growthcurver
Kathleen sprouffske (2018). growthcurver: Simple Metrics to Summarize Growth Curves. R package version 0.3.0.

lme4
Douglas Bates, Martin Maechler, Ben Bolker, Steve Walker (2015). Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software, 67(1), 1-48. doi:10.18637/jss.v067.i01.

lmerTest
Kuznetsova A, Brockhoff PB, Christensen RHB (2017). "lmerTest Package: Tests in Linear Mixed Effects Models." _Journal of Statistical Software_, *82*(13), 1-26. doi: 10.18637/jss.v082.i13 (URL: https://doi.org/10.18637/jss.v082.i13).

MASS
Venables, W. N. & Ripley, B. D. (2002) Modern Applied Statistics with S. Fourth Edition. Springer, New York. ISBN 0-387-95457-0

nortest
Juergen Gross and Uwe Ligges (2015). nortest: Tests for Normality. R package version 1.0-4.

RColorBrewer
Erich Neuwirth (2014). RColorBrewer: ColorBrewer Palettes. R package version 1.1-2.

tibble
Kirill Müller and Hadley Wickham (2020). tibble: Simple Data Frames. R package version 3.0.0.

tidyr
Hadley Wickham and Lionel Henry (2020). tidyr: Tidy Messy Data. R package version 1.0.2.

vegan
Jari Oksanen, F. Guillaume Blanchet, Michael Friendly, Roeland Kindt, Pierre Legendre, Dan McGlinn, Peter R. Minchin, R. B. O'Hara, Gavin L. Simpson, Peter Solymos, M. Henry H. Stevens, Eduard Szoecs and Helene Wagner (2019). vegan: Community Ecology Package. R package version 2.5-6.

All packages are available under https://CRAN.R-project.org/.

9.9 Preliminary experiments leading up to the protocol

For the earliest experiments (first two columns in Table 13) one whole male fly was crushed in 50 µL sterile 1X PBS using a pestle. Later experiments (same table) used 2 males, that were crushed in 100 µL sterile 1X PBS. A dilution series ranging from 1:1, 1:10, 1:100 to 1:1000 was made and 10 µL homogenate were plated on each medium.

During the colony streak test the order of the media was changed for each colony. Afterwards the growth of the colonies on different plate types was analyzed. Although most colonies grew on all types of media during the first two experiments, this was only possible for an average of 30 % in the next experiments (see Table 13). Combined with colonies that grew on both mannitol and tryptic soy, an average of 87 % of total colonies were able to grow on both of these media. The key factor of this shift seems to be MRS, because more than half of the colonies originally cultured on this medium were not able to grow on it, although they could grow on the other two media.

Table 13: Counts of all colonies, that grew on one type of agar (column header) and were then streaked on all three types of agar (row header): Bottom row: count of colored colonies; For the first two columns only one individual fly was used.

	Original culture conditions							
	oxic	**anoxic**	**oxic**			**anoxic**		
	Tryptic soy	**Tryptic soy**	**MRS**	**Tryptic soy**	**Mannitol**	**MRS**	**Tryptic soy**	**Mannitol**
Mann,Tryp, MRS	60	65	33	46	31	12	26	29
Mann,Tryp	4	22	55	35	15	29	21	17
Mann,MRS			12	1	1	1		6
Tryp,MRS			2		1	6		
Mann			1		5	1	1	1
Tryp				3			2	
MRS			1			3		
None			1			1		
Sum	64	87	105	85	53	53	50	53
Colored	3	4	24	5	3	0	22	29

Table 13 also shows that, unlike the first time, bacteria was grown on tryptic soy under anoxic conditions, in the later experiment slightly less than half of the colonies were colored. The variety of bacteria seems to be higher on anoxic mannitol plates than on oxic ones. For MRS agar this was the other way around. Additionally, only one fourth of colonies from MRS plates were colored compared to around half on the other two media.

The colored colonies form patterns that are described in Table 14. For example, a large number of colonies were brown when grown on MRS plates, but only colonies that originated from MRS cultures were yellow on this type of medium. Other yellow colonies either didn't grow on MRS agar or had a brown color. The same phenotype was observed for orange colonies, although these were less abundant. Another interesting pattern were prismatic-like, transparent colonies on mannitol agar, that only occurred on plates originating from mannitol cultures. No specific color was found solely on tryptic soy plates.

Table 14: All color patterns observed during these culturing experiments: Black columns indicate colonies, that did not grow.

Mannitol	Tryptic soy	MRS	Quantity
white	white	brown	22
white		brown	7
beige		brown	6
transparent	white	brown	6
yellow	yellow		6
beige	white	brown	5
beige	white		5
orange	orange		5
yellow	yellow	brown	5
	beige		5
white	white	yellow	4
transparent	white		3
white	yellow		3
beige	white	white	1
beige	white	yellow	1
beige		white	1
orange	orange	brown	1
white	beige		1
white	white	beige	1
white	yellow	white	1
yellow	white	white	1

After the first trial run the four colonies, that were only able to grow on MRS agar (see Table 14), were identified as *Lactobacillus* and preserved.

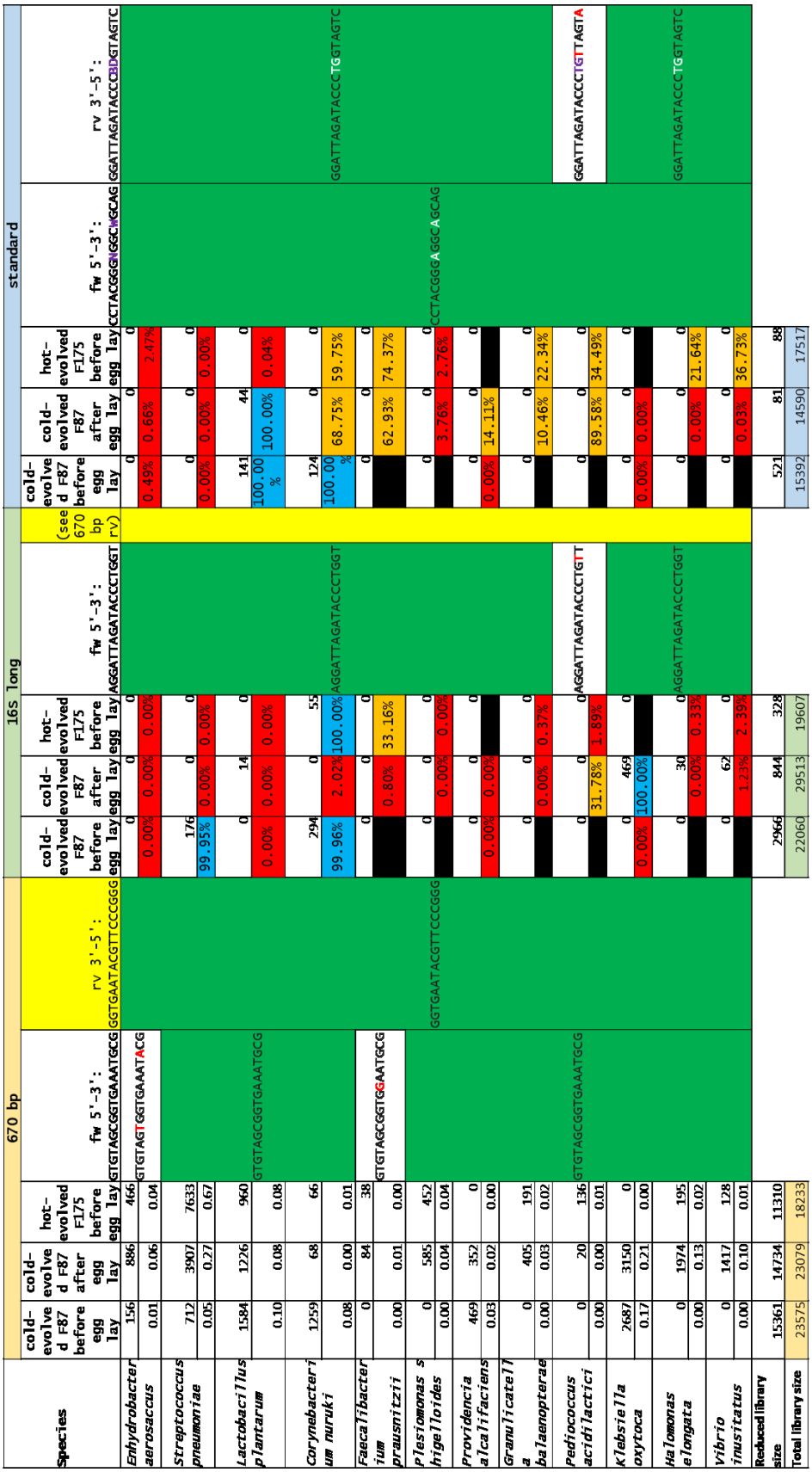
9.10 Primer comparison

Table 15: Overview of the microbiome samples analyzed at the genus level using the three primer pairs: 670 bp, 16S long and IPG's standard

Genus	Cold						Hot		
	F87						F175		
	1			2			1		
	670 bp	16S long	stand-ard	670 bp	16S long	stand-ard	670 bp	16S long	stand-ard
Wolbachia	0	13523	13541	0	17733	12567	2	17258	16908
Acetobacter	0	4431	453	0	8519	688	0	1860	0
Streptococcus	712	176	0	3907	0	0	7633	0	0
Serratia	7137	994	0	0	0	0	0	0	0
Klebsiella	2687	0	0	3150	469	0	0	0	0
Lactobacillus	1584	0	141	1226	14	44	960	0	0
Swaminathania	0	91	741	0	872	1246	0	130	508
Halomonas	0	0	0	1974	30	0	195	0	0
Corynebacterium	1259	294	124	68	0	0	66	55	0
Vibrio	0	0	0	1417	62	0	128	0	0
Enhydrobacter	156	0	0	886	0	0	466	0	0
Plesiomonas	0	0	0	585	0	0	452	0	0
Pseudomonas	0	0	119	0	0	37	685	0	54
Providencia	469	0	0	352	0	0	0	0	0
Stenotrophomonas	600	0	110	0	0	0	0	0	0
Erwinia	0	696	0	0	0	0	0	0	0
Granulicatella	0	0	0	405	0	0	191	0	0
Citrobacter	504	0	0	0	0	0	0	0	0
Leuconostoc	0	0	20	0	88	0	268	0	0
Morganella	0	366	0	0	0	0	0	0	0
Candidatus Portiera	253	82	0	0	0	0	0	0	0
Sediminibacterium	0	24	0	0	61	0	0	223	0
Marinobacter	0	0	0	284	0	0	0	0	0
Gluconobacter	0	6	123	0	116	0	0	13	0
Desemzia	0	139	0	0	109	0	0	0	0
Rhodanobacter	0	0	0	177	0	0	0	0	0
Pediococcus	0	0	0	20	0	0	136	0	0
Staphylococcus	0	0	0	155	0	0	0	0	0
Yersinia	0	145	0	0	0	0	0	0	0
Faecalibacterium	0	0	0	84	0	0	38	0	0
Acidiphilium	0	15	0	0	93	0	0	11	0
HTCC2207	0	0	0	0	0	0	69	0	0
Bradyrhizobium	0	0	0	0	11	0	0	50	0
Bacillus	0	50	0	0	0	0	0	0	0
Burkholderia	0	0	7	0	0	0	0	0	34
Thermoanaero-bacterium	0	0	0	0	0	0	23	0	0
Prevotella	0	0	0	19	0	0	0	0	0
Desulfotomaculum	0	0	0	11	0	0	0	0	0
Flavobacterium	0	0	0	11	0	0	0	0	0
Planctomyces	0	0	0	3	0	0	0	0	0
Flavisolibacter	0	0	0	0	0	0	0	1	0
Percentage of assigned reads	65.2	95.3	99.9	63.8	95.5	99.9	62.0	100.0	99.9
Library size	23575	22060	15392	23079	29513	14590	18233	19607	17517

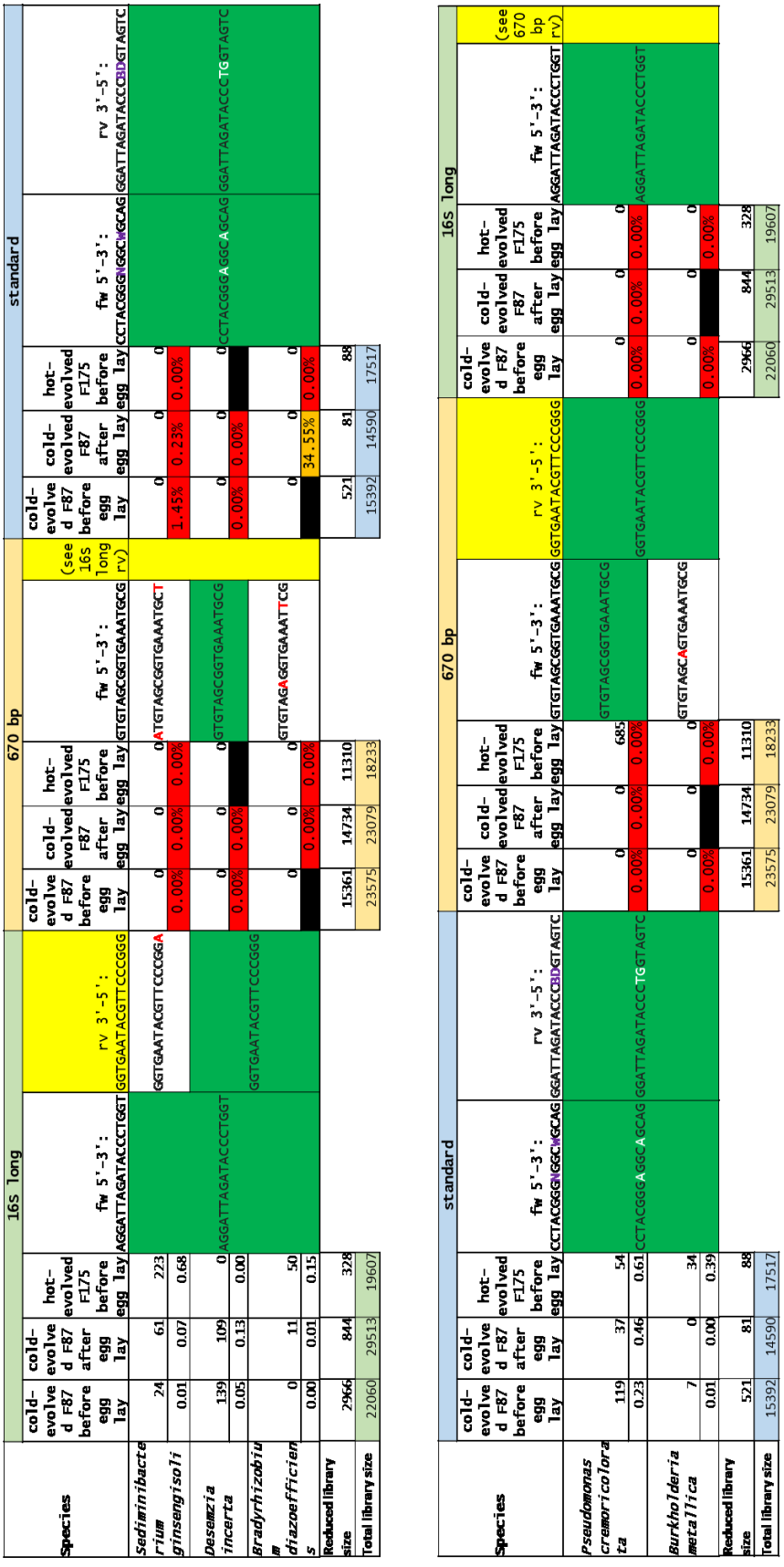
	670 bp				16S long				standard			
Species	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': GTGTAGCGGTGAAATGCG	rv 3'-5': GGTGAATACGGTTCCCGGG	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': AGGATTAGATACCCTGGT	(see 670 bp rv)	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': CCTACGGGNGGCWGCAG	rv 3'-5': GGATTAGATACCCBDGTAGTC
wolbachia wMel	0	0	2	GTGTAGAGGTGAAATTCG	GGTGAATACGTTCTCGGG	13523	17733	17258			13541	12567	16908	CCTACGGGAGGCAGCAG	
	0.00	0.00	0.00			0.61	0.60	0.88			0.88	0.86	0.97		
Swaminathania salitolerans					GGTGAATACGTTCCCTGG										
	0	0	0			4431	8519	1860			453	688	0	CCTACGGGAGGCAAGCAG	
	0.00	0.00	0.00			0.00	0.03	0.01			0.05	0.09	0.03		
Acetobacter indonesiensis									AGGATTAGATACCCTGGT						GGATTAGATACCCTGGTAGTC
	0	0	0			91	872	130			741	1246	508		
	0.00	0.00	0.00	GTGTAGAGGTGAAATTCG		0.20	0.29	0.09			0.03	0.05	0.00		
Gluconobacter sphaericus					GGTGAATACGTTCCCGGG									CCTACGGGAGGCAGCAG	
	0	0	0			6	116	13			123	0	0		
	0.00	0.00	0.00			0.00	0.00	0.00			0.01	0.00	0.00		
Acidiphilium iwatense															
	0	0	0			15	93	11			0	0	0		
	0.00	0.00	0.00			0.00	0.00	0.00			0.00	0.00	0.00		
Library size	23575	23079	18233			22060	29513	19607			15392	14590	17517		

Table 16: Sequence alignment of primers and the 16S rRNA gene of example species of the orders Rickettsiales and Rhodospirillales; Count and percentage of total reads are also depicted.



Species	670 bp				16S long						standard			
	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': GTGTAGCGGTGAAATGCG	rv 3'-5': GGTGAATACGTTCCCGGG	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': AGGATTAGATACCCTGGT	(see 670 bp rv)	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': CCTACGGGNGGCWGCAG	rv 3'-5': GGATTAGATACCCBDGTAGTC
Enhydrobacter aerosaccus	156	886	466	GTGTAGTGGTGAAATACG		0	0	0			0	0	0		
	0.01	0.06	0.04			0.00%	0.00%	0.00%			0.49%	0.66%	2.47%		
Streptococcus pneumoniae	712	3907	7633	GTGTAGCGGTGAAATGCG		176	0	0			0	0	0		
	0.05	0.27	0.67			99.95%	0.00%	0.00%			0.00%	0.00%	0.00%		
Lactobacillus plantarum	1584	1226	960			0	14	0			141	44	0		
	0.10	0.08	0.08			0.00%	0.00%	0.00%			100.00%	100.00%	0.04%		
Corynebacterium nuruki	1259	68	66			294	0	55			124	0	0		
	0.08	0.00	0.01			99.96%	2.02%	100.00%			100.00%	68.75%	59.75%		
Faecalibacterium prausnitzii	0	84	38	GTGTAGCGGTGGAATGCG		0	0	0			0	0	0		
	0.00	0.01	0.00			0.00%	0.80%	33.16%			0.00%	62.93%	74.37%		
Plesiomonas shigelloides	0	585	452			0	0	0			0	0	0		
	0.00	0.04	0.04			0.00%	0.00%	0.00%			0.00%	3.76%	2.76%		
Providencia alcalifaciens	469	352	0			0	0	0			0	0	0		
	0.03	0.02	0.00			0.00%	0.00%	0.00%			0.00%	14.11%	0.00%		
Granulicatella balaenopterae	0	405	191			0	0	0			0	0	0		
	0.00	0.03	0.02			0.00%	0.00%	0.37%			0.00%	10.46%	22.34%		
Pediococcus acidilactici	0	20	136	GTGTAGCGGTGAAATGCG		0	0	0	AGGATTAGATACCCTGTT		0	0	0		GGATTAGATACCCTGTTAGTA
	0.00	0.00	0.01			0.00%	31.78%	1.89%			0.00%	89.58%	34.49%		
Klebsiella oxytoca	2687	3150	0			0	469	0			0	0	0		
	0.17	0.21	0.00			0.00%	100.00%	0.00%			0.00%	0.00%	0.00%		
Halomonas elongata	0	1974	195			0	30	0	AGGATTAGATACCCTGGT		0	0	0		GGATTAGATACCCTGTAGTC
	0.00	0.13	0.02			0.00%	0.00%	0.33%			0.00%	0.00%	21.64%		
Vibrio inusitatus	0	1417	128			0	62	0			0	0	0		
	0.00	0.10	0.01			0.00%	1.23%	2.39%			0.00%	0.03%	36.73%		
Reduced library size	15361	14734	11310			2966	844	328			521	81	88		
Total library size	23575	23079	18233			22060	29513	19607			15392	14590	17517		

Table 17: Sequence alignment of primers and the 16S rRNA gene using reduced library sizes (library size without Rickettsiales and Rhodosprillales); reference: primer 670 bp; Count, percentage of total reads and binomial distribution probability are also depicted.



Species	16S long				670 bp					standard					
	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': AGGATTAGATACCCTGGT	rv 3'-5': GGTGAATACGTTCCCGGG	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': GTGTAGCGGTGAAATGCG	(see 16S long rv)	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': CCTACGGGNGGCWGCAG	rv 3'-5': GGATTAGATACCCBDGTAGTC
Sediminibacterium ginsengisoli	24	61	223	GGTGAATACGTTCCCGGA		0	0	0	ATGTAGCGGTGAAATGCT		0	0	0		
	0.01	0.07	0.68			0.00%	0.00%	0.00%			1.45%	0.23%	0.00%		
Desemzia incerta	139	109	0	AGGATTAGATACCCTGGT	GGTGAATACGTTCCCGGG	0	0	0	GTGTAGCGGTGAAATGCG		0	0	0	CCTACGGGAGGCAGCAG	GGATTAGATACCCTGTAGTC
	0.05	0.13	0.00			0.00%	0.00%				0.00%	0.00%			
Bradyrhizobium diazoefficiens	0	11	50			0	0	0	GTGTAGAGGTGAAATTCG		0		0		
	0.00	0.01	0.15			0.00%	0.00%	0.00%				34.55%	0.00%		
Reduced library size	2966	844	328			15361	14734	11310			521	81	88		
Total library size	22060	29513	19607			23575	23079	18233			15392	14590	17517		

Species	standard				670 bp					16S long					
	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': CCTACGGGNGGCWGCAG	rv 3'-5': GGATTAGATACCCBDGTAGTC	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': GTGTAGCGGTGAAATGCG	rv 3'-5': GGTGAATACGTTCCCGGG	cold-evolved F87 before egg lay	cold-evolved F87 after egg lay	hot-evolved F175 before egg lay	fw 5'-3': AGGATTAGATACCCTGGT	(see 670 bp rv)
Pseudomonas cremoricolorata	119	37	54	CCTACGGGAGGCAGCAG	GGATTAGATACCCTGTAGTC	0	0	685	GTGTAGCGGTGAAATGCG		0	0	0	AGGATTAGATACCCTGGT	
	0.23	0.46	0.61			0.00%	0.00%	0.00%		GGTGAATACGTTCCCGGG	0.00%	0.00%	0.00%		
Burkholderia metallica	7	0	34			0	0	0	GTGTAGCAGTGAAATGCG		0	0	0		
	0.01	0.00	0.39			0.00%	0.00%	0.00%			0.00%		0.00%		
Reduced library size	521	81	88			15361	14734	11310			2966	844	328		
Total library size	15392	14590	17517			23575	23079	18233			22060	29513	19607		

Table 18: Sequence alignment of primers and the 16S rRNA gene using reduced library sizes (library size without Rickettsiales and Rhodosprillales); references: 16S long (left) and standard (right); Count, percentage of total reads and binomial distribution probability are also depicted.

9.11 Growth experiments

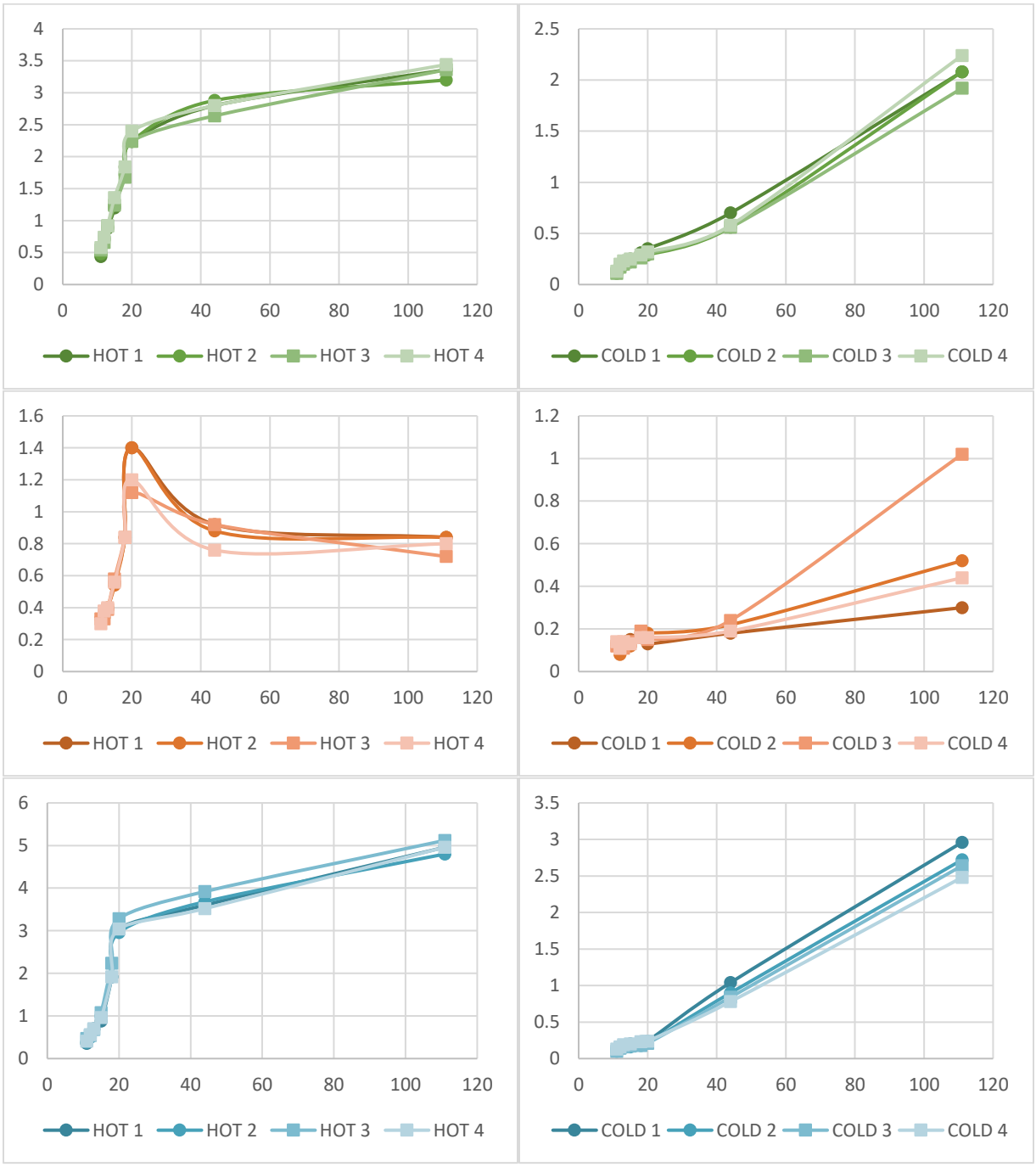


Fig. 13: Mannitol (green), MRS (orange) and tryptic soy (blue) graphs of the same liquid culture (*L. plantarum*); 4 separate eprouvettes per medium

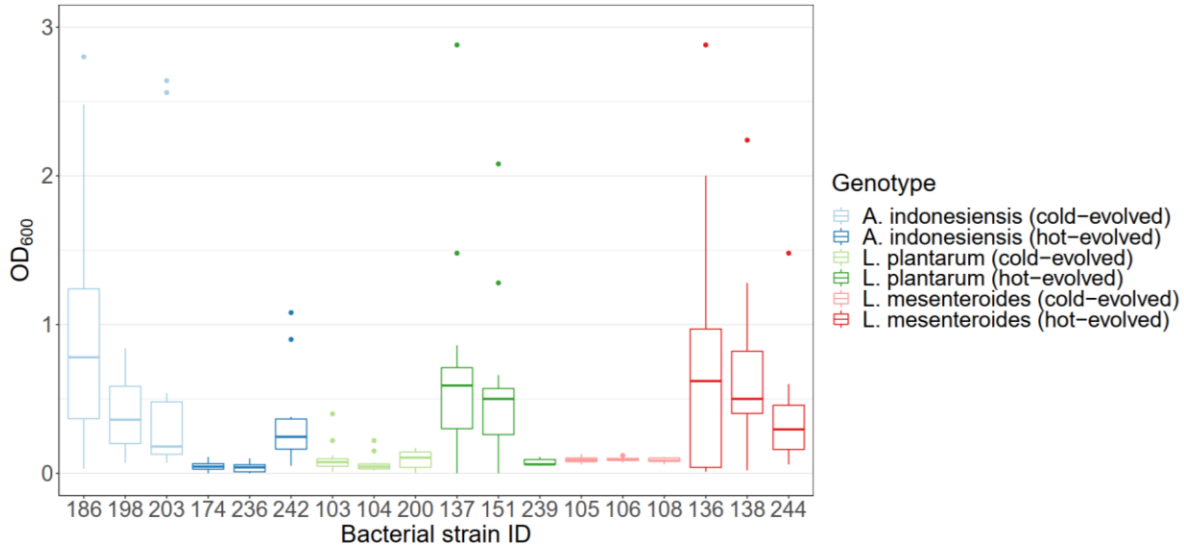


Fig. 14: Boxplots of 12 h measurement points combining all media and culturing temperatures from both timetables

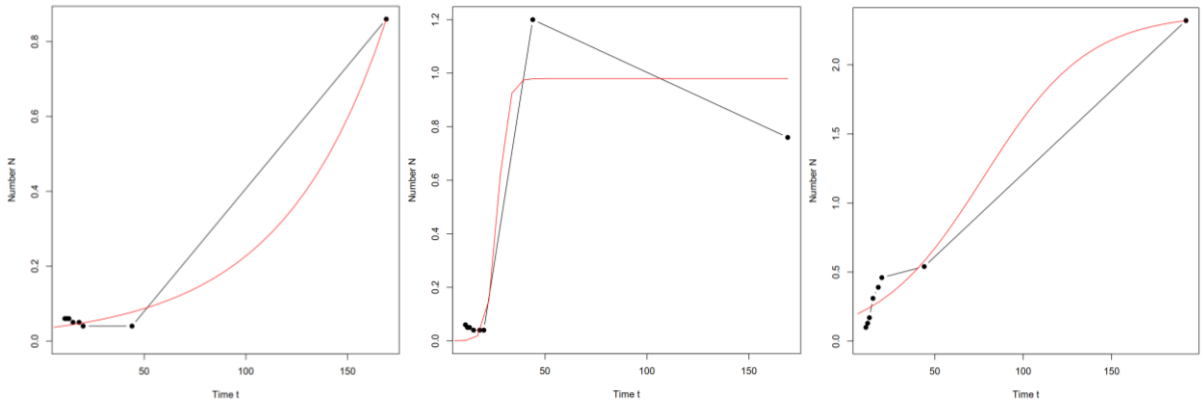


Fig. 15: Examples of growth curves generated that have a large fitting error or do not cover the exponential phase: *L. plantarum* strain 239 (hot-evolved) mannitol 20 °C day regime (left), *L. plantarum* strain 239 (hot-evolved) tryptic soy 28 °C night regime (middle), *A. indonesiensis* strain 203 (cold-evolved) MRS 28 °C night regime (right)

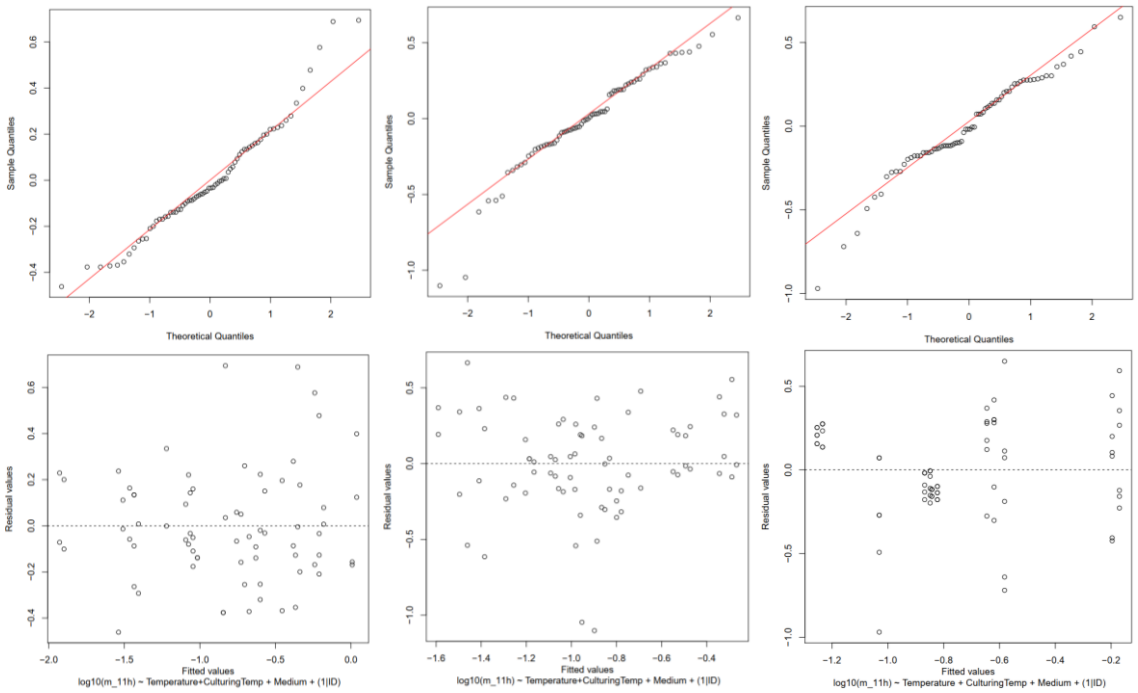


Fig. 16: Q-Q (top) and homogeneity plots (bottom) made for the LMERs of *A. indonesiensis* (left), *L. plantarum* (middle) and *L. mesenteroides* (right)

9.12 Cultured microbiome of *D. simulans* strains

Overview of cultured fly populations

Table 19: Fruit fly populations, that were in culturing experiments

Origin	T (°C)	Culture	Colony streak test		Culturing conditions
		Time (days)	T (°C)	Time (days)	
Portugal	28/18	1-2	37	2	mannitol, tryptic soy; an- and oxic
South Africa	15	2	20, 37	1	mannitol, tryptic soy; oxic
Florida	28/18	2-4	28/18	2	mannitol, tryptic soy; an- and oxic
Portugal	28/18	2-4	28/18	2	mannitol, MRS, tryptic soy; an- and oxic

Portugal (18-28 °C) cultured at 37 °C

Table 20: Sequenced colonies of crushed whole flies of *D. simulans* from Portugal reared at 18-28 °C but cultured at 37 °C, quantified first in total as well as the percentage thereof, and then further categorized as aerobic and anaerobic bacteria.

	Total	Aerobic	Anaerobic
Enterococcus wangshanyuanii	13	7	6
Enterococcus faecalis	12	6	6
Dermacoccus nishinomiyaensis & D. barathri	5	1	4
Lactobacillus plantarum	2	1	1
Leuconostoc mesenteroides & L. pseudomesenteroides	2	1	1
Corynebacterium nuruki	1	0	1
Micrococcus luteus	1	1	0
Morganella morganii	1	0	1
Staphylococcus epidermidis	1	1	0
Sum	38	18	20

Portugal (18-28 °C) cultured at 18-28 °C

Table 21: Sequenced colonies of crushed whole flies of *D. simulans* from Portugal reared at 18-28 °C, quantified first in total as well as the percentage thereof, and then further categorized as aerobic and anaerobic bacteria.

	Total	Aerobic	Anaerobic
Enterococcus faecalis	7	2	5
Corynebacterium nuruki & C. provencense	6	0	6
Lactobacillus	6	2	4
Acetobacter indonesiensis	4	4	0
Micrococcus	4	4	0
Leuconostoc	3	0	3
Paenibacillus	3	3	0
Morganella morganii	2	0	2
Pseudoclavibacter	1	0	1
Sum	36	15	21

Florida 5 (18-28 °C) cultured at 18-28 °C

Table 22: Sequenced colonies of crushed whole flies of *D. simulans* from Florida reared at 18-28 °C, quantified first in total as well as the percentage thereof, and then further categorized as aerobic and anaerobic bacteria.

	Total	**Aerobic**	**Anaerobic**
Acetobacter	13	6	7
Corynebacterium nuruki	8	3	5
Lactobacillus	6	3	3
Leuconostoc	2	1	1
Lysinibacillus	2	1	1
Paenibacillus	2	1	1
Staphylococcus epidermidis	1	0	1
Sum	34	15	19

Hot- and cold-evolved fly samples from Florida

Table 23: Sequenced colonies of crushed whole flies of cold- and hot-evolved *D. simulans* from Florida

	Total	**cold-evolved F87 before egg lay**	**cold-evolved F88 before egg lay**	**hot-evolved F175 before egg lay**	**hot-evolved F175 after egg lay**
Acetobacter	44	12	17	6	9
Leuconostoc pseudom. & L. mesenteroides	26	7	5	8	6
Enterobacter ludwigii & Citrobacter freundii	11	5	6	0	0
Lactobacillus	11	3	1	2	5
Corynebacterium nuruki	6	0	0	4	2
Morganella morganii	6	0	0	5	1
Dermacoccus nishinomiyaensis	3	1	0	0	2
Enterococcus faecalis	3	0	0	2	1
Arthrobacter sulfonivorans & Pseudoarthr. sulf.	2	0	0	2	0
Moraxella osloensis & Enhydrobacter aerosaccus	2	2	0	0	0
Bacillus cereus & B. mycoides & B. anthracis	1	0	0	1	0
Micrococcus	3	0	0	0	3
Paenibacillus xylanilyticus	1	0	0	0	1
Sum	119	30	29	30	30

Colonies of all *D. simulans* populations

Table 24: Combined list of all colonies sequenced from *D. simulans* populations

	Sum	**South Africa**	**Portu-gal 37 °C**	**Portu-gal 18-28 °C**	**Flor-ida 18-28 °C**	**Flor-ida cold-evo-lved F87 before egg lay**	**Flor-ida cold-evo-lved F87 after egg lay**	**Flor-ida hot-evo-lved F175 before egg lay**	**Flor-ida hot-evo-lved F175 after egg lay**
Acetobacter	61	0	0	4	13	12	17	6	9
Corynebacterium	21	0	1	6	8	0	0	4	2
Lactobacillus	27	2	2	6	6	3	1	2	5
Leuconostoc	40	7	2	3	2	7	5	8	6
Lysinibacillus	2	0	0	0	2	0	0	0	0
Paenibacillus	6	0	0	3	2	0	0	0	1
Staphylococcus	4	2	1	0	1	0	0	0	0
Morganella morganii	9	0	1	2	0	0	0	5	1
Enterococcus faecalis	22	0	12	7	0	0	0	2	1
Enterococcus wangshanyuanii	13	0	13	0	0	0	0	0	0
Bacillus cereus, B. mycoides & B. anthracis	1	0	0	0	0	0	0	1	0
Moraxella osloensis & Enhydrobacter aerosaccus	4	2	0	0	0	2	0	0	0
Enterobacter ludwigii & Citrobacter freundii	15	4	0	0	0	5	6	0	0
Dermacoccus nishinomiyaensis & D. barathri	8	0	5	0	0	1	0	0	2
Micrococcus	8	0	1	4	0	0	0	0	3
Pseudoclavibacter	1	0	0	1	0	0	0	0	0
Arthrobacter sulfonivorans & Pseudoarthr. sulf.	2	0	0	0	0	0	0	2	0
Sum	244.0	17	38	36	34	30	29	30	30