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Effects of gut-associated yeasts on Drosophila melanogaster performance

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Graduate Program in Biology

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Abstract

I used *Drosophila melanogaster* as a model to study the role of the gut microbiota, specifically yeasts, in animal physiology. I used *Saccharomyces cerevisiae*, the yeast commonly included in *Drosophila* diet, and *Lachancea kluyveri*, isolated from some *Drosophila* in the wild, and generated axenic (germ-free) and gnotobiotic (yeast-fed) flies. I found that *L. kluyveri* persists in the crop, as ascospores and vegetative cells, longer than *S. cerevisiae*. Some *L. kluyveri* vegetative cells survive passage through the gut. Egg to adult development time is reduced by 14% in vials containing live *L. kluyveri* or *S. cerevisiae*, whereas heat-killed yeasts reduced development time by 3.5-4.5%. Chill coma recovery time was decreased from 27 to 17 minutes by live *L. kluyveri*, but not heat-killed yeast. I conclude that there is a biological interaction between *D. melanogaster* and gut yeast, and that this system is suitable to explore the role of gut-associated yeasts on animal physiology.

Keywords

Gut microbiota, *Lachancea kluyveri, Saccharomyces cerevisiae*, chill coma recovery time, development time, persistence, yeast sporulation, interindividual variation
Co-Authorship Statement

This work was conducted with the mentorship and financial support of Dr. Brent Sinclair and Dr. André Lachance. All aspects of sampling design and analysis were planned in cooperation with Drs. Sinclair and Lachance, and any publications subsequent to the completion of this work will be co-authored with them.
Acknowledgments

First, I would like to thank my supervisor Dr. Brent Sinclair for the wonderful opportunity of being part of his lab. It all started with a springtail image in the home page of the Sinclair’s lab website, and two years later I have gained so much, not just in my academic career but also in my personal development, none of which would have been possible without the constant support and encouragement from my supervisor. Thank you Brent for all your dedication and guidance throughout my degree.

I am incredibly fortunate to have such an amazing family that has supported me every step of the way. To my wonderful husband, Andrew, I could never thank you enough for all your patience, love, and dedication you have bestowed upon our son and me so that I could devote a great portion of my life to achieving my academic goals. I would also like to thank my terrific son, Martín, for all his help and wisdom beyond his age during difficult times. I will always be thankful to my parents for giving the wonderful gift of experiencing and loving nature – not all kids get to have a pet goat and talk to iguanas in their backyard!

I also wish to thank Dr. André Lachance, my advisor and teacher, for his endless patience, knowledge, and time throughout my degree. Thank you for listening to my endless questions and showing me many of the techniques necessary for the completion of my Master’s project…and welcoming me to ‘live’ in your lab for the past two years. I thank Dr. Jim Staples, member of my advisory committee for his enthusiasm and advice in developing my project. I would also like to thank Dr. Ben Rubin for all his help with statistical analyses.

I am also thankful to all the Sinclair team. I feel exceptionally lucky to have been surrounded by a group of the most enthusiastic bright people I have ever met. Their love for science is genuinely inspiring. Thank you all for the knowledge-filled and emotional support.

All of this work would have been impossible to accomplish without the help of amazing volunteers and work-study students, which took on the arduous task of preparing fly food, fly wrangling, and data collection (even when it meant working at 4 °C on a Sunday!). Thank you Anika Mcbean, Lauren Linseman, Ami Patel, SongMin Lee, and Niam Kataria.
Last but not least, I want to thank Dr. Hugh Fackrell, my undergrad thesis supervisor for giving me a first taste of what research means. Thank you for your mentorship and guidance through uncertain times and for having faith in my potential. I wouldn’t be where I am now if it weren’t for you.
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1 Introduction

Animals host an abundant and varied community of microorganisms. These microbes form complex interactions with each other and their host, and while some can cause disease, the vast majority contribute to the organism’s health (McFall-Ngai et al., 2013). The collection of all the microbes cohabiting an animal is termed the microbiome (Lederberg & McCray, 2001) or microbiota (Lederberg, 2004). Both terms have been used interchangeably, with the most recent tendency being to describe the microorganism community as the microbiota and their collective genome as the microbiome (Bäckhed et al., 2005). I will use this terminology throughout my thesis.

The microbiota is present in the epithelial and mucosal surfaces of animals such as skin, digestive, respiratory and urogenital tracts (Chiller et al., 2001; Tlaskalová-Hogenová et al., 2011). These microbial communities are composed of bacteria, fungi, archaea, protista, and viruses (Dillon & Dillon, 2004; Ganter, 2006; Nam et al., 2008). In animals, the greatest number of microorganisms is found in the digestive tract (Whitman et al., 1998; Douglas, 2015) and these can affect nutrition, immunity, and behaviour (McFall-Ngai et al., 2013). In addition to extracellular microbes, there are a number of intracellular microbes, most of which are bacteria, which are best known in insects (Gil et al., 2004). Most of what is known about host-microbiota interactions has been the result of studies conducted with bacteria as symbionts, but little is understood about the role of the other components of the gut microbiota such as fungi. Yeasts are found in the gut of most animals (Abranches, 1998; Ganter, 2006), and for that reason, have the potential to affect their host physiology. I have manipulated the gut microbiota of vinegar flies, *Drosophila melanogaster* Meigen, to assess the effects of yeasts as possible symbionts on the physiology of their host.

1.1 The microbiota

Most animals live in close association with an array of microorganisms. Microbes can colonize animal surfaces such as the skin, fur or exoskeleton as well as the internal organs like lungs and intestines (McFall-Ngai et al, 2013), even the cytoplasm of
specialized cells. Symbiosis, in its most general definition, describes organisms that live together, which includes microbes and their host (Oulhen et al., 2016). The interactions in a symbiotic relationship can be further classified as mutualistic (both species benefit), commensal (one species benefits while the other one is unaffected) or parasitic (one species benefits at expense of the other) (McFall-Ngai, 2013). Adding to the complexity, there is a special type of symbiosis where the symbiont has evolved to inhabit specialized cells in its host.

The microbiota of an animal is complex, composed of multiple species of microbes fulfilling multiple functions. For example: bacteria from the phyla Firmicutes, Proteobacteria, and Bacteriodetes (Jami & Mizrahi, 2012), methanogenic classes of archaea (Brulc et al., 2009), and fungi in the genus Orpinomyces and Anaeromyces (Sirohi et al., 2013) all aid the digestion of plant material in ruminants. Lower termites and wood-feeding cockroaches depend on flagellated protozoa and bacteria to break down cellulose and provide nitrogen (Desai & Brune, 2012). The bioluminescent bacterium Vibrio fisheri is necessary for the development and functionality of the bobtail squid’s light organ (Montgomery & McFall-Ngai, 1998). Some microbes are essential to the development of their host’s immune system (Naik et al., 2012) while others are co-opted by parasitoid wasps to weaken the immune system of their prey (Shelby & Webb, 1999). Despite the different groups of microorganisms found in persistent association with animals, bacteria have been most intensively studied (Chandler et al., 2012).

Microorganisms are widespread on an animal’s body, from epithelial surfaces to specialized cells. The lungs, previously thought to be sterile in healthy individuals, are colonized by bacteria important for the development of allergen tolerance in mice (Gollwitzer et al., 2014). Microbes are also found on the skin of healthy animals, and this symbiosis promotes immunity against cutaneous pathogens (Naik et al., 2012). Mycetocytes are specialized cells inhabited by microbes such as the intracellular bacteria Buchnera and Wolbachia which are essential for the normal development and reproduction of aphids (Moran, 2007), and many other arthropods (Dillon & Dillon, 2004). Likewise, the gastrointestinal tract is host to an abundance of microbes, many of which are responsible for the maintenance of their host’s fitness.
1.1.1 Gut microbiota

While there are microbes living in virtually every part of an animal’s body, the gut contains some of the most abundant and complex microbiota. For instance, in humans the colon contains approximately 70% of all the microorganisms in the body. Similarly, the hindgut of most insects contains the largest numbers of microbes (Douglas, 2015). The gut microbiota affects animal physiology including metabolic functions and immune and nervous system development (See Table 1.1). Furthermore, the gut microbiota influences brain development and behaviour linked to stress and pain modulation in mice (Cryan & O’Mahony, 2011).

Ingesting live cultures of beneficial microorganisms can replicate or enhance the effects of the gut microbiota on an animal. Probiotics are live microbes that when ingested provide beneficial effects to the host by improving or restoring the gut microbiota (Fuller, 1989). The microorganisms most used as probiotics belong to the bacterial genera *Lactobacillus* and *Bifidobacterium*, and the yeast species *Saccharomyces boulardii* (Pham et al., 2008). These are commonly used in the treatment and prevention of antibiotic-associated or infectious diarrhoea in mammals (Otero, 2006; Bybee et al., 2011). Other gastrointestinal disorders such as Crohn’s disease, traveller’s diarrhoea, and necrotizing enterocolitis can potentially be treated with probiotics (Sazawal et al., 2006). Use of probiotics is also becoming a common practice in aquaculture because it enhances productivity and disease resistance in fish (Nayak, 2010). Poultry farming has also benefited by the use of probiotic supplements in animal feed (Wolfenden & Hargis, 2014). Probiotics enhance growth and immune responses against pathogens, reducing the need for antibiotics (Khan & Naz, 2013).
Table 1.1 – Function of the gut microbiota in different hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Symbiont</th>
<th>Proposed function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle, sheep</td>
<td>Bacteria, fungi, methanogenic archaea, bacteriophages</td>
<td>Digestion of plant cell wall</td>
<td>Klieve &amp; Bauchop, 1998 Brulc et al. 2009</td>
</tr>
<tr>
<td>Pigs, rodents</td>
<td>Bacteria</td>
<td>Promotion of gut immune system development</td>
<td>Rothkötter et al., 1991 Schokker et al., 2015</td>
</tr>
<tr>
<td>Rats</td>
<td>Bacteria</td>
<td>Regulation of hepatic bile acid and cholesterol absorption</td>
<td>Wostmann, 1973</td>
</tr>
<tr>
<td>Mice</td>
<td>Bacteria</td>
<td>Reduce colon inflammation, increase longevity, reduce anxiety levels, and motor activity</td>
<td>Matsumoto et al., 2011 Cryan &amp; O’Mahony, 2011</td>
</tr>
<tr>
<td>Hoatzins</td>
<td>Bacteria</td>
<td>Degradation of plant material</td>
<td>Godoy-Vitorino et al., 2008</td>
</tr>
<tr>
<td>Turtles, tortoises, iguanas</td>
<td>Bacteria, methanogenic archaea</td>
<td>Fermentation of plant material</td>
<td>Yuan et al., 2015</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Bacteria</td>
<td>Controls development and protein uptake</td>
<td>Bates et al., 2006</td>
</tr>
<tr>
<td>Lower termites</td>
<td>Bacteria, flagellates</td>
<td>Nitrogen and nutrient provisioning</td>
<td>Hongoh et al., 2008 Desai &amp; Brune, 2012</td>
</tr>
<tr>
<td>Sea urchins</td>
<td>Bacteria</td>
<td>Cellulose digestion and provision of essential amino acids</td>
<td>Fong &amp; Mann, 1980</td>
</tr>
<tr>
<td>Nematodes</td>
<td>Bacteria</td>
<td>Nutrient provision</td>
<td>Cabreiro &amp; Gems, 2013</td>
</tr>
</tbody>
</table>
1.1.1.1 Effects of the gut microbiota on insect hosts

Insects have a less diverse microbial community than vertebrates, which facilitates the manipulation and study of their gut microbiota (Pernice et al., 2014). Microbial symbionts in the gut can aid the host by providing nutritional supplements, promoting growth and mating success, detoxifying the host’s food source and protecting the animal against pathogenic microbes (Engel & Moran, 2013).

Insects with nutrient-poor diets such as sap, blood, and wood require a gut microbiota capable of breaking down food components to provide essential nutrients. These nutrients in turn decrease development time and increase body mass and mating success. Herbivorous insects such as platapid stinkbugs (Hosokawa et al., 2006), common house crickets (Acheta domestica) (Kaufman & Klug, 1991), wood-feeding Cryptocercus cockroaches (Urbina et al., 2013), lower termites (Desai & Brune, 2012), and bark beetles (Dendroctonus rhyzophagus) (Morales-Jiménez et al., 2012) depend on the microbial fermentation of complex plant carbohydrates, such as lignocellulose, for the production of volatile fatty acids. Insects that feed on vertebrate blood such as triatomine kissing bugs, responsible for Chagas disease transmission, require vitamin B biosynthesized by their gut microbiota (Beard et al., 2002). All these insects develop more slowly and have low reproductive success when their normal gut microbiota is perturbed (dysbiosis) (Engel & Moran, 2013).

Insects are also presented with the challenge of overcoming plant toxins and pesticides. The coffee berry borer (Hypothenemus hampei) and the cabbage maggot (Delia radicum) are able to feed on plants toxic to most other insects. In both cases, the insects’ gut microbiota produces enzymes able to break down toxins into smaller, harmless compounds (Ceja-Navarro et al., 2015; Welte et al., 2016). The gut microbes of pest insects such as the bean bug (Riptortus clavatus) and the oriental chinch bug (Calvelerius saccharivorus), a pest of sugarcane, have the ability to degrade the commonly used insecticide fenitrothrin (Kikuchi et al., 2012).
Gut microbes also defend their insect host against pathogens. Bumble bees (Crithidia bombi) that acquire their gut microbiota from the feces of their nest mates have lower parasite loads when compared to those reared in isolation (Koch & Schmid-Hempel, 2011). In a similar manner, the gut microbiota of the desert locust (Schistocerca gregaria) and the greater wax moth (Galleria mellonella) protect the hosts from infection by reducing the pathogen load through the production of narrow spectrum bactericides such as bacteriocins (Jarosz, 1979; Dillon et al., 2005). Other possible mechanisms by which the gut microbiota inhibits pathogen growth is competition for resources and adhering surfaces (Dillon et al., 2005), as well as modification of physiological conditions within the gut by the production of lactic acid by Lactobacillus and Bifidobacterium spp. (Forsgren et al., 2010).

1.1.1.2 Drosophila melanogaster and its gut-microbes as a system to study host-microbe interactions

Drosophila melanogaster is a suitable organism for the study of host-microbial interactions because of the molecular, genetic, and physiological tools available, short generation time, and tractability in the lab (Chandler et al., 2012). Also, the gut microbiota of D. melanogaster has a low diversity of bacteria and yeast symbionts. The bacterial microbiota of Drosophila is mostly composed of three taxonomic groups: Acetobacteraceae, Enterobacteriaceae, and Lactobacillales (Wong et al., 2011; Chandler et al., 2011). Yeast species recovered from the gut of wild-caught Drosophila spp. belong in their majority to the genera Saccharomyces, Hanseniaspora, and Hansenula, while other yeast species such as Candida, Kluyveromycs, and Lachancea are found in lower numbers (Phaff et al., 1956; El. Tabey Shehata et al., 1955; Lachance et al., 1995, Chandler et al., 2012). Most of the known effects of the microbiota in Drosophila have been determined by studying the effects of bacteria as gut symbionts, while yeasts have received less attention (Hoang et al., 2015).

Gut bacteria affect the metabolism, immune response, and behaviour of D. melanogaster. Lactobacillus plantarum and Acetobacter pomorum can influence growth and development in flies through the production of acetic acid and branched-chain amino
acids used in the insulin-signaling pathway (Shin et al., 2011; Storelli et al., 2011). In addition, the gut microbiota provides vitamin B riboflavin and protein, and suppresses excessive lipid storage by female D. melanogaster (Wong et al., 2014). All these metabolic effects are usually observable in nutrient-poor conditions (Douglas, 2009). Resident gut bacteria also protect the flies from infection by the pathogen Candida albicans and stimulate epithelial renewal and stem cell proliferation in the gut (Cronin et al., 2009; Buchon et al., 2009; Glittenberg et al., 2011). By comparing gene expression between axenic and conventionally reared flies, Broderick et al. (2014), demonstrated that the gut microbiota affects the expression of genes involved not only in immune response, but gut morphology as well, and that many of these effects are dependent on the immune deficiency (Imd) pathway. The gut microbiota of D. melanogaster might also maintain basal levels of gut epithelia renewal through the activating the JAK–STAT (Janus kinase–signal transducers and activators of transcription) and JNK (c-Jun NH2 terminal kinase) pathways (Buchon et al., 2009).

Bacteria in the gut can even influence mate choice in D. melanogaster; females are more likely to copulate with males reared on the same media and the effect is eliminated in flies reared under axenic conditions (Sharon et al., 2010; 2011). Bacterial gut microbiota might be responsible for modifying the composition of cuticular hydrocarbon sex pheromones as antibiotic treatment reduced the levels of these hydrocarbons (Sharon et al., 2010). The microorganisms responsible for physiological effects on the host have not always been identified, and, as with L. plantarum and A. pomorum – both affecting development, multiple species might contribute to the same response through the same or different mechanism (Shin et al., 2011; Storelli et al., 2011). All the microorganisms (bacteria, yeasts, archaea and protists) commonly found in the gut of healthy animals might contribute to the host’s fitness, but the contribution of each group remains unknown.

Finally there is some evidence that gut microbes fundamentally change the physiological state of their hosts. For example, A. pomorum affects D. melanogaster development and body size by activating the flies’ insulin/insulin-like growth factor signaling (IIS) through the production of acetic acid by the pyrroloquinoline quinone–
dependent alcohol dehydrogenase (PQQ-ADH)–dependent oxidative respiratory chain of
the bacterium (Shin et al., 2011). Shin et al., (2011) also found evidence that the activity
of PQQ-ADH of A. pomorum maintains basal levels of intestinal stem cells and epithelial
renewal in D. melanogaster via the activation of the JAK-STAT pathway. In some cases,
the contribution of the gut microbiota to host physiology is only observed in nutrient poor
diets. L. plantarum promotes D. melanogaster larval growth by enhancing assimilation of
protein from the food, which in turn activates TOR kinase activity in the fat body and
prothoracic gland responsible for controlling growth rate and the duration of growth
phase respectively (Storelli et al., 2011).

1.2 Drosophila-yeast interactions

Yeasts are commonly found in the gut of Drosophila in nature. Drosophila spp.
disperse yeasts to new habitats as they seek yeast-rich substrates where to feed and
oviposit (Starmer & Fogleman, 1986). Yeasts are an important component of most
Drosophila diet, both in wild and laboratory populations, providing nutrients such as
vitamin B, amino acids, sterols, and fatty acids (Anagnostou et al., 2010). The
Drosophila-yeast interaction is not species-specific, as ecological and geographical
factors have a prominent role in determining the composition of the yeast community in
the gut. For example, Drosophila spp. caught in eastern North America have higher yeast
species diversity in their gut than flies caught in western North America (Lachance et al.,
1995). Nonetheless, the diet of the flies seems to be the most important determinant for
gut-yeast composition. For example, Candida panamericana recovered from mushroom
feeding flies is rarely found in association with cactus or sap feeders (Lachance et al.,
1995; Chandler et al., 2012).
Table 1.2 – Yeast species isolated from the gut of various *Drosophila* species and their presumed diet. Yeast genera: *Candida*, *Hensiniaspora*, *Pichia*, *Kluveromyces*, *Lachancea*, and *Saccharomyces*

<table>
<thead>
<tr>
<th>Yeast genera</th>
<th>C. amapae</th>
<th>C. diversa</th>
<th>C. panamericana</th>
<th>C. restingae</th>
<th>H. occidentalis</th>
<th>H. uvarum</th>
<th>P. kluvieri</th>
<th>P. kudriavzevi</th>
<th>P. heedii</th>
<th>L. thermotolerans</th>
<th>L. lactis</th>
<th>L. kluyveri</th>
<th>L. montanus</th>
<th>Komagataella pastorianum</th>
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<tbody>
<tr>
<td><strong>Fruit-feeders</strong></td>
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<td><em>D. hydei</em></td>
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<td><em>D. immigrans</em></td>
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<td><em>D. sulfurigaster</em></td>
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<tr>
<td><em>D. malerkotliana</em></td>
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Lachance *et al.*, 1995; Chandler *et al.*, 2012
Drosophila acquire their gut yeasts by feeding on substrates colonized by those yeasts. Volatile chemicals produced by yeasts attract both adults and larvae, for example D. suzukii and D. melanogaster prefer species of yeasts most commonly found within their gut (Scheidler et al., 2015). These yeast species are also the yeasts that best support growth, development, and survival (Starmer et al., 1990; Anagnostou et al., 2010). Interestingly, the food preference differs between adults and larvae, and female flies often oviposit on substrates that benefit the adult and not the larvae (Cooper, 1960). The yeast most commonly used in the diet of Drosophila laboratory populations is S. cerevisiae (Hoang et al., 2015); however, this yeast is rarely found associated with flies in nature and flies do not prefer S. cerevisiae over yeast species recovered from wild flies (Chandler et al., 2012).

Yeasts affect Drosophila physiology and immunology. Drosophila spp. deprived of yeast in their diet have shorter lifespans and slower developmental rates (Anagnostou et al., 2010; Ebbert et al., 2003). D. melanogaster reared on low yeast diets accumulate more lipids and have reduced fecundity (Skorupa et al., 2008). Stress tolerance in Drosophila is also affected by the presence of yeast. For example, D. melanogaster fed on a diet of live S. cerevisiae had increased survival after exposure to 0 °C for 16 h, fungal infection (Beauveria bassiana) and starvation when compared to those without yeast in their diet (Le Rohellec & Le Bourg, 2009). Live S. cerevisiae also increases the immune response in D. melanogaster parasitized by the wasp Leptopilina boulardi (Vass & Nappi, 1998).

Yeasts also affect mate choice and mating success by modifying female behaviour in D. melanogaster (McRobert, 1986) and in D. buzzatii, increasing the attractiveness of males whose larger body size is the result of the yeast in their diet during development (Norry & Vilardi, 1996). Yeast cells are transmitted between flies during courtship in Drosophila buzzatii (Starmer et al., 1988), and in D. subobscura, males present yeast-rich droplets as nuptial gifts (Steele, 1986).
Previous studies have considered the *Drosophila*-yeast interaction as a form of mutualism in which the only contribution of the yeast to the fly is as a food source (Douglas, 2009; Starmer & Lachance, 2011; Blum et al., 2013), but the role of yeast as a gut microbe remains largely unexplored. Yeasts can survive and replicate within the gut of the *D. melanogaster* (Bakula, 1969), suggesting the potential for yeasts to be part of the resident gut microbiota. To understand the complexity of *Drosophila*-microbial interactions, the role of yeasts in the gut microbiome must be reconsidered.

Determining the *Drosophila* phenotypes that are affected by the gut microbiota is necessary for understanding the contribution of these microbes, including yeasts, to their host. While the mechanisms by which gut microbes affect phenotype are not well understood, the gut microbiota affects *D. melanogaster* development (Anagnostou et al., 2010), growth (Storelli et al., 2011), and physiology (Sommer & Bäckhed, 2013). To study the effects of gut associated yeasts on *D. melanogaster* physiology, I chose two phenotypes: development time from egg to adult, and chill coma recovery time (CCRT). Yeasts and bacteria both can affect development in *D. melanogaster*, therefore contributing to overall fitness, but the contribution of yeasts is considered solely nutritional (Anagnostou et al., 2010; Shin et al., 2011).

CCRT is one of a suite of partially correlated measurements of cold tolerance in *Drosophila*, and can be used to determine the geographic distributions limits of *Drosophila* species (Andersen et al., 2015). At the onset of chill coma (cold induced paralysis), Na$^+$ and water gradually move from the hemolymph to the hindgut (MacMillan et al., 2012). The water and ion imbalance causes the concentration of K$^+$ in the hemolymph to increase, leading to cell injury and death (MacMillan & Sinclair, 2011b). Chill coma recovery is accompanied by the reversal of this process where sodium ions and water homeostasis in the hemolymph is re-established (MacMillan et al., 2012). Recovery from chill coma is metabolically demanding as indicated by the increase in metabolic rate during recovery in the fall field cricket (*Gryllus pennsylvanicus*) (MacMillan et al., 2012). Furthermore in *D. melanogaster*, supplementation of the diet with NaCl, KCl, and sucrose helps maintain K$^+$ balance in the hemolymph; flies that maintain this balance recover faster from chill coma (Yerushalmi et al., 2016).
Starvation tolerance is another phenotype affected by yeast, while the mechanisms are not understood; the effect of the yeast is believed to be nutritional (Le Rohellec & Le Bourg, 2009). While live yeast might also affect starvation resistance, this is not examined in depth within my thesis. Effects on starvation might be the result of gut yeasts affecting metabolism and energy use.

1.3 Methods for studying the gut microbiota

Manipulation of the gut microbiota of an animal is necessary to understand the contribution and function of the microorganisms and their host. While simplification of the system by studying the interaction between the host and a single or few microbes is not an accurate representation of nature, it allows researchers to explore the function of each partner and isolate the mechanism mediating the interaction. There are several ways to study the role of the gut microbiota. One approach is to eliminate members of the gut microbial community selectively via the administration of antibiotics (Ben-Yosef et al., 2008). While this is an easy method to remove gut microbes, it does not fully eliminate the microbial community and as a result one cannot discount possible interactions with resident microbes that are resistant to the specific antibiotic (Koga et al., 2007). Also the use of antibiotics can have other physiological effects such as reducing body protein content and fecundity (Ridley et al., 2013). In vertebrates, another way to explore the effects of the gut microbiota on physiology is through fecal transplants (Turnbaugh et al., 2009). This technique introduces new microbes to an already-existing community, and therefore does not differentiate between effects from specific microbes and the interaction between the introduced and resident microbiota (Van den Abbeele et al., 2013). A more controlled approach is to rear axenic and gnotobiotic animals and compare the effect of specific members of the gut microbiota (Smith et al., 2007).

Most microbiota studies involve the comparison of conventionally reared animals and their axenic (germ-free) counterparts. Specific pathogen free (SPF) and gnotobiotic (when all microbes present are identified) animals are also models to study the effects of specific microorganisms on the host (Smith et al., 2007). Microbes are characterized by culture-dependent techniques where microorganism colonies are grown in Petri dishes on species-specific media and culture-independent 16S ribosomal RNA (rRNA) sequencing.
is used to identify microorganisms not able to grow outside the host (Smith et al., 2007; Wong et al., 2013). Culture-independent microbial identification only targets bacteria, and it does not detect the presence of yeasts in the gut.

Rearing axenic animals is essential for studying the microbiota’s function. Small mammals such as rodents (Smith et al., 2007), miniature pigs (Haverson et al., 2007), and dogs (Cohn & Heneghan, 1991) have to be delivered by caesarean section under sterile conditions. The animals are then raised in flexible film isolators with HEPA filters and all materials including water and feed entering the enclosure have to be autoclaved (Smith et al., 2007). Chickens can also be reared axenically by submerging the fertilized eggs in a bleach solution before incubation in a similar isolator (Cheled-Shoval et al., 2014). Fish embryos are soaked in a solution containing a mixture of antibiotics and rinsed in a sodium hypochlorite solution before transferring them to sterile flasks containing embryo media (Bates et al., 2006). In a similar manner, axenic Drosophila are reared from eggs sterilized by submerging them in a sodium hypochlorite solution, followed by ethanol submersion. This treatment removes the chorion, and the embryos are then transferred to sterile vials containing fly food media (Brummel et al., 2004). Other insects like pea aphids are reared axenically with antibiotic treatments (Douglas, 1998). The development of axenic animals delivered through caesarean and reared in sterile conditions is not always successful and it requires extensive training and expensive equipment (Smith et al., 2007). In addition, there is a limit on the number of animals that can be reared in an isolator and contamination due to faulty seals can drastically reduce the sample size (Smith et al., 2007; Cheled-Shoval et al., 2014). Because of their smaller size, insects such as aphids and flies can be kept in larger numbers, and while contamination is still possible, their generation time is usually shorter than that of mammals, making insects a good system for the study of the gut microbiota.

1.4 Objectives

The overall goal of my thesis was to determine the effects of gut yeasts on Drosophila melanogaster phenotype. I accomplished this by addressing the following objectives:
1. **Determine if yeasts survive and replicate in the gut of *D. melanogaster***. I measured persistence of *S. cerevisiae* and *L. kluyveri* cells collected from dissected guts of flies and evaluated the structural integrity of the cells using fluorescent and phase-contrast microscopy.

2. **Identify *D. melanogaster* physiological traits affected by gut-associated yeasts**. I reared axenic and gnotobiotic (with live yeast in their gut) flies. I then measured development time and CCRT in both populations to determine the effect of yeast on a life history trait and on the tolerance to cold stressors.

3. **Determine whether the effects of yeasts on fly physiology are due to nutrition or host-microbe interactions**. I compared development and CCRT in axenic and gnotobiotic *D. melanogaster* with flies that remained axenic but received different concentrations of heat-killed yeast as a nutritional supplement.
2 Methods

2.1 Fly rearing

The *D. melanogaster* population I used was established in 2007 from individuals collected in London, Ontario, Canada (43°00’ N, 81°15’ W; Marshall & Sinclair, 2010). I reared the flies at standard densities (ca. 50 eggs/vial; non-overlapping generations) and conditions (21.5 ± 1 °C, 60 ± 5 % relative humidity, 13 h:11 h L:D), in 35 mL glass vials containing 10 mL autoclaved Tucson fly food (1 L dH2O, 45 g sugar, 30 g cornmeal, 18 g dry active yeast (Fleischmann’s Yeast, Farinex, QC, Canada), 12 g agar; adapted from Markow & O’Grady, 2006). I transferred adult flies to small acrylic egg collection cages (⌀ = 3.5 cm, h = 5.8 cm), capped with Petri dishes (⌀ = 3.5 cm) filled with grape/apple agar (100 mL fruit juice, 100 mL dH2O, 3 g agar; adapted from Merkling & Rij, 2015) topped with yeast paste (active dry yeast hydrated with dH2O to form a smooth paste) to stimulate oviposition. After three days of changing the agar plates with active yeast every 24 h, the agar plates were replaced with fresh ones without active yeast. The flies were allowed to lay eggs overnight before egg collection.

2.2 Surface sterilization of *D. melanogaster* eggs to grow axenic flies

The environment within *D. melanogaster* eggs is free of microbes and the embryos do not acquire their gut microbiota until they emerge and feed on the chorion (Bakula, 1969). While it is possible to create axenic flies by fully removing the chorion, I have demonstrated that surface sterilization of the eggs is enough to grow axenic flies without the added stress of full dechorionation (Figures 2.1). I surface-sterilized the *Drosophila* eggs by submerging them in 70 % ethanol and gently moving them with a sterile inoculation loop for 5 min. I transferred the eggs to sterile nylon filters (20 µm pore) and rinsed them with autoclaved phosphate-buffered saline (PBS) until all ethanol was removed. I transferred the eggs onto a thin layer of food media (1.5 g agar, 1.5 g active yeast, 4.3 g sugar, 100 mL dH2O) and then into autoclaved glass vial containing autoclaved Tucson food to a density of 50 eggs per vial. The vials remained undisturbed under standard conditions until the flies reached adult stage. All steps were performed in
a laminar flow cabinet using aseptic techniques to minimize contamination. See appendix A for a step-by-step protocol.

I sampled each vial to confirm the axenic state of adult flies before all experiments. I selected three flies from each vial (72 h after eclosion) by introducing CO₂ into the vials through a sterile nozzle and removing the flies with sterile forceps. Each fly was homogenized in sterile saline using a tissue grinder and plated on Yeast-Malt medium (YM) to verify axenic state. YM agar (1 % glucose, 0.5 % peptone, 0.3 % malt extract, 0.3% yeast extract, 2 % agar) is a nutrient-rich medium that favours the growth of yeasts, moulds, and acidoduric bacteria (Zimbro et al., 2009). I confirmed the axenic state of the flies by the lack of microbial colonies on the YM plate after 48 h incubation at 25 °C (Figure 2.2).

I also tested the flies’ axenic state by conducting polymerase chain reactions (PCR) with adult D. melanogaster (one fly per vial, for a total of 12 vials) using rRNA primers Pro341F: 5’-CCTACGGGNGGCASCAG-3’ and Pro805R: 5’-GACTACNVGGGTATCTAATCC-3’ for the detection of bacteria and archaea (Takahashi et al., 2014). I homogenized each fly in 220 µL of water using a tissue grinder and plated each homogenate (10 µL/plate) on YM agar and LB agar (1 % Tryptone, 0.5 % yeast extract, 1.5 % agar) to test for microbial growth. I performed a Phenol:Chloroform (1:1) DNA extraction following the protocol used in Lachance et al. (2016). Each PCR reaction contained 5.0 µL Template and a final concentration of 1X PCR Buffer, 0.2 mM (each) dNPT mixture, 1.5 mM MgCl₂, 0.2 µM (each) primer mix, 1.0 unit Platinum® Taq DNA Polymerase as per Invitrogen’s protocol. The cycling parameters I used were as follows: 35 cycles, denaturation (92 °C for 10 s), annealing (53 °C for 15 s – minus 0.1°C/cycle), and extension (72 °C for 20 s plus 1 s/cycle).

The resulting agarose gel electrophoresis revealed the presence of a band identified by DNA sequencing as Staphylococcus epidermidis (Fig. 2.3). While these results suggest contamination of the axenic flies, YM and LB agar plates inoculated with the fly homogenates showed no microbial growth after 48 h of incubation at 25 °C. S. epidermidis readily grows on both media (Gustafsson et al., 2003). Only samples five and
seven showed microbial growth (one colony each) after ten days of incubation. YM and LB agar plates inoculated with food sampled from each vial showed no microbial growth even after ten days. Although the PCR results suggested contamination of the axenic *D. melanogaster* I used as my study, the bacterium is not likely to have affected fly physiology because it was present in very small numbers (1 CFU/vial found in two of 12 vials sampled). Furthermore, vials of the axenic flies were randomly selected for each treatment (See section 2.5), minimizing any possible effect derived from the contamination.

**Figure 2.1—*Drosophila melanogaster* eggs.** Eggs washed with sterile PBS (A) maintain an intact chorion as indicated by the breathing appendages (arrow). Surface-sterilizing the eggs with 70 % ethanol for 5 min (B) does not remove the chorion. Full dechorionation of *Drosophila* eggs with 0.6 % bleach (C), a commonly used method for generating axenic flies.
Figure 2.2 – YM agar plates inoculated with whole adult *D. melanogaster* homogenized in sterile PBS. Adult flies grown from eggs washed in sterile PBS (A) show yeasts (arrow) and bacterial colonies (circle), while adult flies grown from eggs washed in 70 % ethanol (B) show no microbial colonies.

Figure 2.3 – PCR analysis of *D. melanogaster* homogenates. Each numbered sample represents an individual fly from a different vial. I used bacterial DNA (*Serratia marcescens*) as a positive control. Samples 1, 3 and 10 were negative for microbial DNA. From samples 2 and 5, which had the more intense bands, only sample 5 had one colony growing in LB agar after ten days. None of the plates inoculated with food from the vials showed microbial growth.
2.3 Yeast cultures

I introduced either of two species of yeast to the axenic flies as a meal 48 h before testing or into the media where the eggs were placed if testing for development time. I chose *Saccharomyces cerevisiae* because it is used as part of *Drosophila* diet in many laboratories, but there is not good evidence that it is commonly associated with *Drosophila* spp. in nature (Hoang et al., 2015). Due to unforeseen circumstances, I obtained *Lachancea kluyveri* instead of the yeast I had requested (*Pichia kluyveri*), a yeast frequently recovered from the gut of many *Drosophila* species (Lachance et al., 1995; Chandler et al., 2012). I continued using *L. kluyveri* since the first persistence experiments were promising (See results. *Lachancea kluyveri* was originally isolated from the crop of *D. pinicola* in the Yosemite region of California (Phaff et al. 1956). It has also been recovered from other *Drosophila* species in the Pinery Provincial Park, Ontario, Canada (Lachance et al., 1995).

To culture *S. cerevisiae*, I suspended a pellet of active dry yeast (Fleischmann’s Yeast, Farinex, QC, Canada) in sterile PBS and plated the suspension on a Petri dish containing YM-agar. I incubated the plate at 25 °C for 48 h and isolated a single colony by streaking a new plate with it. All subsequent *S. cerevisiae* used during my project were obtained from a culture derived from this originally isolated colony, with the exception of the GFP-labelled strain. Dr. P. Lajoie provided the *S. cerevisiae* (strain W303) expressing green fluorescent protein (GFP) which was also kept as a culture at 25 °C on YM agar plates. *L. kluyveri* (strain NRRL Y-12651) was obtained from the yeast culture collection of the Biology Department, University of Western Ontario (UWOPS) and kept as a culture on YM agar plates at 25 °C for the length of my project. I obtained a fresh culture of *L. kluyveri* (strain NRRL Y-12651) from UWOPS a year later, when the original yeast used as a treatment in my experiments was no longer affecting *D. melanogaster* phenotype due to repeated subculturing (See sections 2.6 and 3.2).

To transfer the yeasts into the fly vials, I prepared yeast suspensions by removing colonies from the YM agar with an inoculation loop and fully mixing them in sterile PBS. I determined the concentration of each suspension using standard curves calibrated to number of cells and absorbance (560 nm wavelength and 1 cm light path). I pipetted the
yeast suspension (10 µL) into the sterile vials of containing 10 mL of Tucson food before transferring the adult flies or the fly eggs for the developmental time experiments.

2.4 Persistence of yeasts in the gut

Yeasts as microorganisms have the capacity to interact with their host in ways other than as nutritional components. For this interaction to take place, the yeast would need to be alive and either reside within the gut or be a transient symbiont. I assessed how long yeast persists inside the gut of *D. melanogaster* by determining how long viable cells can be recovered from the gut of flies after the original yeast source has been removed.

2.4.1 *Drosophila melanogaster* yeast consumption

Since *Drosophila* develop their gut microbiota by ingesting microorganisms (Bakula, 1969), I verified that the flies ate yeast. I separated male and female adult axenic flies into population cages (300 flies/cage). The population cages were made of 3.8 L PET squared plastic jars (23 cm × 15 cm × 13 cm) sterilized with 70 % ethanol closed with a clipped autoclaved medical stockinette for easy access. I fed the flies by placing a Petri (⌀ = 100 mm) dish with banana media (1 L water, 112.5 g banana, 47.5 g corn syrup, 30 g barley malt, 27.5 g active yeast, 3 mL propionic acid, 2 g methylparaben) inside the cage (Markow & O’Grady, 2006) and added 50 µL of a thick paste made with either live *S. cerevisiae* or *L. kluyveri* dyed with 50 µL of Trypan blue (0.4 %) shaped into a circle (⌀ = 20 mm) (Figure 2.4). The cages were incubated under standard conditions and monitored periodically for 12 h. The diameter of the yeast paste was measured and the flies were surface sterilized and dissected. The blue dye facilitated the comparison between the amount of food consumed by males and females during the same time intervals and had the added advantage of being visible through the abdomen of the fly.
2.4.2 Testing persistence of yeasts through Drosophila serial transfers

I transferred axenic flies (30 flies/vial, 48 h post-eclosion) to sterilized glass vials containing cornmeal medium inoculated with either S. cerevisiae or L. kluyveri and allowed the flies to eat for 24 h. At the end of the 24 h, I transferred the flies to autoclaved Tucson food vials with no further addition of yeast. The transfer was repeated every 24 h, and with each transfer a sample of three flies was taken for microbial load evaluation. I surface-sterilized the sample flies with 70 % ethanol and dissected their
guts. The guts were individually homogenized in 50 µL of sterile PBS. I plated 10 µL of each homogenate on YM agar plates and incubated them at 25 °C for 48 h. Transfers to new vials stopped once there were no longer yeast cells recovered from the sample flies.

I also dissected and plated individual *Drosophila* guts to determine the variation in quantity of yeast cells recovered in flies from the same vial. I transferred axenic flies (30 flies/vial, 48 post-eclosion) into vials containing Tucson food inoculated with live *L. kluyveri* (10 µL of 1.3×10^8 cells/mL suspension). I allowed the flies to eat the yeast for 24 h and then randomly selected three males and three females from each vial, for a total of four vials. I plated individually homogenized guts (50 µL sterile PBS) on YM agar and counted the colony forming units (CFU) 48 h after incubation at 25 °C.

### 2.4.3 Viability of yeast cells in the *Drosophila* gut

To determine if the yeast cells inside the gut of the fly were alive and reproducing, I surface-sterilized and dissected flies that had been feeding on live yeast (*L. kluyveri*) for 24 h. I wet-mounted the guts on microscope slides and used phase contrast microscopy to determine the structural integrity of the yeast cells. Phase contrast microscopy does not require the use of any dyes or other preparations, minimizing damage to living cells. Live and dead yeast cells have different refractive indexes allowing for easy differentiation (Wiemken *et al.*, 1970). I also fed *D. melanogaster* for 24 h with GFP-*S. cerevisiae*. I surface-sterilized the flies, dissected the guts and wet-mounted them for observation using a compound fluorescence microscope (Axio Imager Z1 with ZEN 2012 software, Carl Zeiss Ltd. Canada). I looked for the presence of newly budding yeast cells to assess if replication was still occurring within the gut of the fly (Robinow, 1975) as well as the integrity of the cell wall and ascospore production (Coluccio *et al.*, 2008). I also collected frass from flies fed with live yeast and phase contrast and fluorescence microscopy to determine the structural integrity of the yeast cells (Coluccio *et al.*, 2008).
2.5 Treatment groups

Before most experiments, I divided the flies reared from axenic eggs of the same generation into three groups. I confirmed the axenic state of the *D. melanogaster* in each vial by testing three randomly selected individual as per section 2.2 and transferring the remaining flies to vials inoculated with either sterile saline (axenic), live yeast (gnotobiotic), or heat-killed yeast (axenic with nutritional yeast). I discarded any vials that tested positive for microbial growth.

I transferred axenic *D. melanogaster* to treatment vials 48 h post-eclosion. I prepared each treatment vial by pipetting 10 µL of either sterile PBS (for flies that remained axenic) or 10 µL of yeast suspensions containing $1.65 \times 10^7$ cells/mL of *Saccharomyces cerevisiae* or *Lachancea kluyveri* (for gnotobiotic flies). To test the nutritional contribution of the yeast on *Drosophila* performance, I transferred axenic flies (48 h post-eclosion) to sterile glass vials containing 10 mL of sterile Tucson food inoculated with 10 µL of a suspension containing heat-killed *S. cerevisiae* or *L. kluyveri* at four different concentrations (1×, 2×, 4×, or 8× the concentration of live yeast, equivalent to $1.3 \times 10^8$, $2.6 \times 10^8$, $5.3 \times 10^8$, or $1.1 \times 10^9$ cells/mL). I prepared the heat-killed yeast by placing the suspension in sealed 1.7 mL microcentrifuge tubes at 60 °C in a heating block (Multi-block heater, 2090, Lab-line Instruments) for 10 (1× and 2× concentrations), 20 (4× concentration), or 30 min (8× concentration). I allowed the heat-killed yeast suspensions to cool to room temperature for 30 min before pipetting into autoclaved Tucson food vials. I confirmed that the yeast treatments were no longer viable by the absence of microbial growth on YM agar plates previously inoculated with 10 µL of each suspension and incubated at 25 °C for 48 (Figure 2.5).
Figure 2.5 – YM agar plates inoculated with treatment suspensions. Sterile PBS (A) for the axenic group. Live Lachancea kluyveri (B) for the gnotobiotic group showing yeast colonies. Heat-killed L. kluyveri at 1×, 2×, 4×, 8× the concentration of live yeast (C - F) for groups that remain axenic but received yeast for its nutritional benefits. Lack of microbial growth verified that all heat-killed treatments were effective. I tested the S. cerevisiae treatments in the same manner.

2.6 Effects of yeasts on chill coma recovery time (CCRT)

Chill coma is a reversible paralysis induced by low temperatures (MacMillan & Sinclair, 2011a). Flies are considered to have recovered from chill coma when they are able to right themselves and stand (David et al., 1998). I compared Drosophila chill coma recovery time between the axenic and gnotobiotic groups to determine whether having live yeast in the gut of the fly affected their responses. I transferred axenic flies (48 h
post-eclosion) to vials of Tucson food inoculated with sterile PBS (10 µL) or live *L. kluyveri* (10 µL of a suspension containing $1.3 \times 10^8$ cells/mL) and allowed them to eat for 48 h. I then moved the flies to empty autoclaved vials, placed them in sealed plastic bags and immersed them into an ice water slurry (0 °C) inside a walk-in cooler (4 °C) for 10 min. Once the flies had stopped moving, I put them on open Petri dishes partially submerged in the ice slurry to separate them by sex and transfer them into 1.7 mL microcentrifuge tubes (5 flies/sex/tube). I then placed the microcentrifuge tubes into sealed plastic bags and submerged them in the ice water slurry for a total exposure of 8 h at 0 °C (Jakobs et al., 2015). The flies were then moved to empty 6-well plates at 21.5 °C and observed for time of recovery. This was done with the help of multiple volunteers who received randomly selected microcentrifuge tubes with coded labels to avoid bias. After recovery, I took sample flies from each treatment; surface-sterilized them and plated their dissected guts individually on YM agar, as described in section 2.4.1. Inoculated plates were incubated at 25 °C for 48 h and inspected for microbial growth. I repeated this experiment with live *L. kluyveri* from the fresh culture (See section 2.3) after the subcultured *L. kluyveri* culture failed to affect *Drosophila* CCRT even after following the same methods (See results).

*Drosophila melanogaster* fed live yeast had a shorter CCRT than axenic flies (See section 3.2.1). I tested whether this effect was influenced by the yeast’s nutritional value by including heat-killed *L. kluyveri* as one of the treatments. I transferred axenic flies (48 h post-eclosion) to vials of Tucson food containing sterile PBS (10 µL), live *L. kluyveri* (10 µL of a suspension containing $1.3 \times 10^8$ cells/mL), and heat-killed *L. kluyveri* (1×, 2×, 4× the concentration of live yeast). I measured the CCRT for all treatment groups and tested the microbial growth following the same procedure as the experiment above. I analyzed the CCRT differences between groups with survival analysis with Log-rank test using GraphPad Prism (version 7.0a for Mac OS X, GraphPad Software, San Diego, California, USA).
2.6.1 Inter-individual variation in CCRT and number of yeast in the gut

The chill coma recovery time varied within treatment and sex (See results). There was also variation in the amount of yeast recovered from the gut of individual flies. I tested whether there is a relation between the two variables by repeating the above experiment measuring recovery time and microbial load per each individual fly. I followed the same procedure, transferring flies to treatment vials, exposing them at 0 °C (8 h), but placing only one fly per well to recover at 21.5 °C. I recorded the CCRT of each fly followed by surface sterilization and gut dissection. I homogenized and streaked single gut homogenates on YM agar plates as described in section 2.4.1. I incubated the plates at 25 °C for 48 h before counting the number of colony forming units (CFU). I analyzed the correlation between CCRT and CFU with Pearson’s product-moment correlation in R (version 3.2.2, R Core Team 2015).

2.7 Effects of yeasts on *D. melanogaster* development time

I measured the development time of *D. melanogaster* as the time it takes from egg collection and surface sterilization to eclosion (Hiraizumu, 1961). I allowed oviposition for 3 h before collecting the eggs under a dissecting microscope to assure only five eggs were placed in each vial. The low number of flies in each vial assures that there is not a density effect on development time (Santos et al., 1994). I surface sterilized the eggs and placed them in vials containing Tucson media inoculated with one of six yeast concentrations: sterile PBS (10 µL), live *L. kluyveri* (10 µL of a suspension containing 1.3×10⁸ cells/mL), and heat-killed *L. kluyveri* at different concentrations (1×, 2×, 4×, 8× the original concentration of live yeast). The vials were incubated at standard conditions and checked twice daily for the first nine days (20 vials/treatment). Once eclosion started, the vials were checked for adults every two hours until 24 h after the last adults emerged. I also compared the development time of flies grown in vials inoculated with live *S. cerevisiae* (10 µL of a suspension containing 1.65×10⁷ cells/mL) or heat-killed *S. cerevisiae* (same concentration). I compared development time between treatments with survival analysis using Log-rank test in GraphPad Prism (Linderman et al., 2012).
2.8 Effects of yeasts on starvation resistance in *D. melanogaster*

I measured resistance to starvation as the time a fly is able to survive without consuming any food (Bubliy & Loeschcke, 2005). I transferred adult flies (48 h post-eclosion) to autoclaved glass vials containing 10 mL of Tucson food inoculated with sterile PBS, live *S. cerevisiae* or live *L. kluyveri*. After 48 h of treatment, I anesthetized the flies with CO$_2$ and transferred individual flies (10 flies/sex/treatment) to sterile vials containing 10 mL of 2 % agar. The agar provided the flies with drinking water to avoid a dehydration effect on survival time. I incubated the vials upside-down at 21.5 °C to prevent the flies from sticking to the agar. Survival was recorded hourly until all flies were dead (Bubliy & Loeschcke, 2005; MacMillan *et al.*, 2009). I compared starvation resistance between both treatments using survival analysis with Log-rank test in GraphPad Prism. Since the difference in *Drosophila* starvation resistance between treatments was not statistically significant (See results), I conducted a one-way ANOVA and power analysis to determine the sample size necessary to detect a statistically significant effect between axenic and gnotobiotic treatments (R, version 3.2.2, R Core Team 2015).
3 Results

3.1 Ingestion and persistence of yeasts in the gut

To determine if yeast cells are able to live and reproduce in the gut of *D. melanogaster*, I tested for the persistence and viability of the yeast cells in the gut of the fly once the original source had been removed.

3.1.1 *Lachancea kluyveri* persists longer than *S. cerevisiae* in the gut of female *D. melanogaster*

I was able to culture both yeast species from all female *D. melanogaster* guts 24 h after their last access to live yeast (3 flies/sex/vial; 3 vials/treatment). Only *L. kluyveri* colonies were present in plates inoculated from female fly guts at the second day transfers (48 h after last access of live yeast). While *S. cerevisiae* was not present after the second day transfers, *L. kluyveri* persisted in female guts up to five days after the original source of yeast was removed. Neither yeast species grew readily in plates inoculated with the guts of *D. melanogaster* males (Fig. 3.1). In the cases where there was yeast growth from male fly guts, the number of colonies was always smaller than that of colonies grown from female guts. In flies fed with live *L. kluyveri*, not only did the number of CFU vary between males and females, but also within members of the same sex despite having been kept in the same vial (Fig. 3.2, 3.3).
Figure 3.1 – YM agar plates inoculated with gnotobiotic *D. melanogaster* guts showing yeast colonies recovered up to five days since ending access to live yeasts. Each plate was inoculated with individually homogenized guts. I fed the flies with either live *L. kluyveri* or live *S. cerevisiae* 24 h before transferring them to sterile Tucson food vials. At the end of each day I transferred the flies to sterile vials and removed sample flies (3 flies/sex) for gut dissections and plating.
Figure 3.2 – Variation in number of *L. kluyveri* cells recovered from the gut of individual gnotobiotic *D. melanogaster*. YM plates inoculated with female *Drosophila* guts (solid symbols) had a higher number of colonies after 48 h incubation at 25 °C than males (open symbols). There was also variation between individuals of the same sex within each vial. Each vial is a replicate of the same treatment containing 10 mL of Tucson food inoculated with 10 µL of live yeast suspension (1.3×10⁸ cells/mL), and each point indicates the number of CFU in a single fly.

Figure 3.3 – *L. kluyveri* colonies growing on YM agar plates inoculated with individually homogenized guts of flies from the same vial. All females had more yeast in their gut than males. The number of CFU also varies significantly between flies of the same sex (vial number 3 from Figure 3.2).
3.1.2 Viability and replication of yeast cells in the gut of *D. melanogaster*

Most yeast cells are digested as they pass through the *Drosophila* gut. Yeast cells recovered from the frass of flies fed live GFP-*S. cerevisiae* appeared more spherical as their cell walls were no longer structurally intact (Figure 3.4–B). Digestion of the cell wall caused the formation of spheroplasts with only the cell membrane to maintain their shape (Kelly & Nurse, 2011). *Lachancea kluyveri* cells ingested by *D. melanogaster* formed ascospores while in the crop. The crop also contained many live cells (Fig 3.6–B). A few cells had undergone digestion; the cell walls were no longer intact and the shape of the cells changed to a more spherical appearance forming spheroplasts (Fig. 3.6–A). Most of the yeast cells in the *Drosophila* hindgut and frass had been digested, but a few cells had survived passage through the digestive tract and were even beginning to divide when collected from the frass (Fig. 3.7–B).

3.1.3 Female *D. melanogaster* ingest more yeast than males

I tested whether males and females ingest the yeast. I provided the flies with similar amounts of highly nutritious banana food topped with a thick paste of live *L. kluyveri* dyed with trypan blue for easy visualization (Fig. 2.4). Female flies were immediately attracted to the yeast and remained at higher numbers at the yeast paste when compared to males (Fig. 3.8). Photographs taken of the food plates at 0, 12 and 24 h from the moment they were available to the flies showed the yeast paste in the females cages fully consumed by the end of the experiment (Fig. 3.9). Inspection of 25 individuals from each cage through a dissecting microscope showed female *D. melanogaster* had distended darkened abdomens while the males did not have this characteristic (Fig. 3.10). I also used fluorescent microscopy to detect the GFP-*S. cerevisiae* inside the crop of males and females to determine if both sexes ingested the yeast (Fig. 3.11). Crops dissected from female *D. melanogaster* were highly distended when compared to males, indicating higher yeast consumption. Males did eat the yeast but only in smaller amounts.
Figure 3.4 – Structure comparison of GFP-*S. cerevisiae* before and after passage through the digestive tract of *D. melanogaster*. Yeast cells from the culture used to feed the flies are dividing and have a smooth continuous appearance (A). Yeast cells recovered from the frass of *D. melanogaster* are spherical and the surface of the cell no longer appears continuous, having dark spots within the membrane (B)
Figure 3.5 – Phase-contrast micrograph of *Lachancea kluyveri* culture. Yeast culture before ingestion by *D. melanogaster*. The cells are healthy with intact cell walls and are dividing (red triangles).

Figure 3.6 – Phase-contrast micrographs of *L. kluyveri* within the crop of *D. melanogaster*. The crop was isolated and wet mounted on a microscope slide. The diagonal lines are muscle fibers. Some cells have already undergone digestion and appear as spheroplasts (A, red circle); however, some yeast cells remained structurally intact (B, triangles). Ascospores were abundant within the crop (A & B, marked by red arrows).
Figure 3.7 – *Lachancea kluyveri* cells recovered from the *Drosophila* hindgut and frass. Most of the yeast cells that reached the hindgut of the fly had been digested (circles) or had formed ascospores (arrows). A few cells were alive and even dividing (triangle) when recovered from the frass.

Figure 3.8 – Number of male and female *D. melanogaster* aggregated at the yeast paste every 30 min. Females flies were more numerous at the yeast paste than males at all recorded times. Each cage contained 300 individuals.
Figure 3.9 – Petri dishes containing banana food topped with a thick *L. kluyveri* paste dyed with trypan blue. Photographs taken just before placing the plates into the cages (0 h) containing 300 male or female flies. Twelve hours after introduction of the food the yeast was more disturbed in the female cage than in the male cage (12 h). At the end of the experiment (24 h), the yeast paste in the female cage was fully consumed. The males did not fully consume the yeast paste.
Figure 3.10 – Sample male and female *D. melanogaster* after feeding on live *L. kluyveri* dyed with trypan blue. Female flies showed enlarged darkened abdomens (A). Darkened or enlarged abdomens were not present in males (B).

Figure 3.11 – Micrographs of *D. melanogaster* crops after feeding on *S. cerevisiae* expressing GFP. Females consume a larger quantity of yeast; the crop is distended with a strong fluorescent signal (A). Males consumed the yeast in smaller amounts, the crops are not distended and the fluorescent signal is as strong as in females (B). Crop of a starved fly (C) used to compare signal intensity between the GFP and the gut’s autofluorescence.
3.2 Effect of gut yeast on chill coma recovery time

3.2.1 CCRT time in axenic and gnotobiotic *D. melanogaster*

I induced chill coma in axenic and gnotobiotic flies (n = 75 flies/sex/treatment) and measured the time each fly took to recover (able to stand on its legs). Female flies fed live *L. kluyveri* before entering chill coma, recovered 10 min faster than their axenic counterparts (Log-rank test, df = 1, χ² = 60.07, p < 0.001; Fig. 3.12-A). The effect of yeast on *D. melanogaster* CCRT is only present in the females; males do not show any significant difference in CCRT between axenic and gnotobiotic treatments (Log-rank test, df = 1, χ² = 0.52, p = 0.472; Fig. 3.12-B).

3.2.2 Effects of dietary yeast on *D. melanogaster* CCRT

To determine if the decrease in CCRT in gnotobiotic females is the result of the dietary value of the yeast, I fed axenic flies with heat-killed *L. kluyveri* (1× and 4× the concentration of live yeast). Females fed with heat-killed *L. kluyveri* recovered from chill coma at the same time as axenic females (Log-rank test, df = 2, χ² = 1.86 p = 0.394), while female *D. melanogaster* fed with live yeast recovered 10 min faster (Log-rank test, df = 3, χ² = 120 p < 0.001; Fig. 3.13-A). CCRT in *D. melanogaster* males was not affected by any of the treatments (Log-rank test, df = 3, χ² = 0.17, p = 0.983; Fig. 3.13-B; Table 3.1).

3.2.3 *Lachancea kluyveri* loss of sporulation by repeated subculturing impairs the ability of the yeast to decrease *D. melanogaster* CCRT

I maintained a culture of *L. kluyveri* at 25 °C for over a year by subculturing (transfer of yeast cells from an old plate to new media). I used this culture of *L. kluyveri* to test for its effects on *D. melanogaster* CCRT. Surprisingly, the previously observed decrease in female *D. melanogaster* CCRT was not replicated, even though all conditions were maintained as in previous experiments. The yeast failed to affect the recovery time of males (Log-rank test, df = 1, χ² = 0.53, p = 0.466; Fig. 3.14-C) and females (Log-rank test, df = 1, χ² = 0.58, p = 0.445; Fig. 3.14-A). Since repeated subculturing of yeasts can
affect their physiology, including the ability to produce spores, I measured *D. melanogaster* CCRT with a newly obtained *L. kluyveri* (strain NRRL Y-12651). The fresh *L. kluyveri* culture decreased the chill coma recovery time of *D. melanogaster* females by 5 min when compared to their axenic counterparts (Log-rank test, df = 1, $\chi^2 = 16.49$, $p < 0.001$; Fig. 3.14-B), while males fed the new yeast recovered at the same time as those in the axenic treatment (Log-rank test, df = 1, $\chi^2 = 3.76$, $p = 0.059$; Fig. 3.14-D).

### 3.2.4 Inter-individual variation

To test whether the amount of gut yeast affected the time it took for *D. melanogaster* females to recover from chill coma, I measured CCRT and counted CFU for each single fly. Females with higher yeast counts recovered sooner than female flies from which no yeast growth was detected on YM agar. There is a negative correlation between CFU and CCRT ($n = 15$; Pearson’s product-moment, $p = 0.026$, df = 13, $r = -0.569$; Fig. 3.15).
Figure 3.12 – CCRT in axenic and gnotobiotic *D. melanogaster*. Female flies (A) fed on live *L. kluyveri* 48 h before entering chill coma recovered 10 min faster than axenic flies (fed on sterile food) (Log-rank test, df = 1, $\chi^2 = 60.07$, p < 0.001). Chill coma recovery time in males (B) did not change in the presence or absence of live yeast (Log-rank test, df = 1, $\chi^2 = 0.52$).
Figure 3.13 – **Effect of live and heat-killed *L. kluyveri* on CCRT.** CCRT in females (A) is positively affected by the presence of live *L. kluyveri*. Flies fed live yeast recovered 10 min faster than axenic flies including those that were fed high concentrations of heat-killed yeast (n= 75; Log-rank test, df = 3, $\chi^2 = 120$ p < 0.001). Males (B) in different treatments showed no difference in CCRT (n= 75; Log-rank test, df = 3, $\chi^2 = 0.17$, p = 0.983; Fig. 3.12-B).
Table 3.1 – Median CCRT (min) of *D. melanogaster* fed live or heat-killed *L. kluyveri*. Live *L. kluyveri* decreases the recovery time from chill coma in females, but not in males. Heat-killed yeast does not affect CCRT in either sex.

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<tr>
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<th>Female</th>
<th>Male</th>
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<td>Heat-killed yeast (4×)</td>
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Feeding *D. melanogaster* with a fresh culture of *L. kluyveri* 48 h before chill coma reduces the recovery time in females. Female (Log-rank test, df = 1, $\chi^2 = 0.58$, $p = 0.445$; A) and male flies (Log-rank test, df = 1, $\chi^2 = 0.53$, $p = 0.466$; C) fed with live yeast from the subcultured *L. kluyveri* recovered from chill coma at a similar time as the axenic ones. However, after feeding the flies with the fresh yeast culture, female flies recovered 5 min faster from chill coma than axenic females (Log-rank test, df = 1, $\chi^2 = 16.49$, $p <0.001$; B). Males showed no difference between flies fed live yeast and axenic males (Log-rank test, df = 1, $\chi^2 = 3.76$, $p = 0.059$; D).
Figure 3.15 – Female *D. melanogaster* with higher CFU count recovered faster from chill coma. Females with higher amounts of yeast in the gut recovered faster than those lacking yeast or having low amounts (Pearson’s product-moment, $p = 0.026$, df = 13, $r = -0.569$).
3.3 Effect of yeasts on *D. melanogaster* development time

*Drosophila melanogaster* reared in vials inoculated with either live *S. cerevisiae* or *L. kluyveri* eclosed 40 h faster than the axenic flies (n = 100 flies/ treatment; Log-rank test, df = 2, $\chi^2 = 123$, p < 0.001) with no significance difference between the two live yeast treatments (Log-rank test, df = 1, $\chi^2 = 0.135$, p = 0.713; Fig. 3.16-A). To assess whether the yeast effect of decreasing development time in *D. melanogaster* was the result of the nutritional contribution of the yeast, I inoculated the vials with heat-killed *S. cerevisiae* and *L. kluyveri*. In both cases, the effect of heat-killed yeast on development time was not as pronounced as the live yeast treatments. *D. melanogaster* fed heat-killed *S. cerevisiae* or heat-killed *L. kluyveri* eclosed 14 h and 11 h earlier than axenic flies respectively (Log-rank test, df = 2, $\chi^2 = 47.38$, p < 0.001; Fig. 3.16-B; Table 3.2).

Because the live yeast can keep replicating inside the vial, providing additional nutrients, I tested the development time of *D. melanogaster* in vials inoculated with increasing concentration of the heat-killed yeast (*S. cerevisiae*: 1× and 2×; *L. kluyveri*: 1×, 2×, 4× and 8× the initial concentration of the live yeasts). Flies reared in vials with heat-killed *S. cerevisiae* eclosed 5 and 15 h earlier than axenic flies depending on the yeast concentration (n = 100 flies/ treatment; Log-rank test, *S. cerevisiae*: df = 3, $\chi^2 = 349$, p < 0.001). Flies reared in vials with heat-killed *L. kluyveri* eclosed 3, 1, 3, and 7 h earlier than axenic flies respectively (n = 100 flies/ treatment; Log-rank test, *L. kluyveri*: df = 5, $\chi^2 = 639$, p < 0.001; Fig. 3.17). In both yeast experiments, *D. melanogaster* reared in vials inoculated with live yeasts eclosed 51 and 53 h earlier than those in the axenic vials (Table 3.3).
Figure 3.16 – Effects of live yeasts and heat killed yeasts on *D. melanogaster* development time. Eclosion time between axenic and gnotobiotic flies (A), both yeast species shortened the flies’ development time by 40 h compared to axenic flies (n = 100 flies/treatment; Log-rank test, df = 1, $\chi^2 = 0.135$, $p = 0.713$). Flies in the heat-killed yeast treatments (B) eclosed 14 h for *S. cerevisiae* and 11 h for *L. kluyveri* earlier than axenic flies (n = 100 flies/treatment; Log-rank test, df = 2, $\chi^2 = 47.38$, $p < 0.001$).
Table 3.2 – Median eclosion time (h) of the *D. melanogaster* reared in the presence or absence of yeast. All flies reared in the vials inoculated with either live or heat-killed yeasts eclosed earlier than those reared without yeast. However, live yeast treatments showed the biggest difference from axenic flies.

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<th><em>L. kluyveri</em></th>
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<td>Heat-killed yeast</td>
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Figure 3.17 – Increased amounts of heat-killed *L. kluyveri* decreased *D. melanogaster* time to eclosion. With each increase in the concentration of dead yeast, the survival curves shift to the left, indicating a faster development time. The shape however, remains similar to that of the axenic flies (n = 100 flies/treatment; Log-rank test, *S. cerevisiae*: df = 3, $\chi^2 = 349, p < 0.001$; *L. kluyveri*: df = 5, $\chi^2 = 639, p < 0.001$).
Table 3.3 – Median eclosion time (h) of *D. melanogaster* reared in vials inoculated with live and heat-killed yeast. Flies reared in vials inoculated with live yeasts eclosed earlier than those reared in sterile vials or vials inoculated with heat-killed yeasts. I did not test the effect of heat-killed *S. cerevisiae* at 4× and 8× concentrations on *D. melanogaster’s* development time.

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<tr>
<th></th>
<th><em>S. cerevisiae</em></th>
<th><em>L. kluyveri</em></th>
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3.4 Effect of yeast on *D. melanogaster* starvation resistance

I tested the effect of feeding yeast to *D. melanogaster* 48 h before submitting them to starvation. Females survived longer than males independently of the presence or absence of yeast (Fig. 3.18). While females fed live *L. kluyveri* before starvation seemed to survive longer than axenic ones, the difference was not statistically significant (n = 10 flies/treatment; Log-rank test, df = 1, $\chi^2 = 0.637$, p = 0.425; Fig. 3.18-A). The sample size was small (n=10 flies/sex/treatment), using these data, I conducted a power analysis with significance level = 0.05 and power = 0.8. The sample size necessary for detecting a significant effect with this parameters is n = 26. Starvation resistance in males was not affected by the presence or absence of yeast in their food prior to starvation (n = 10 flies/treatment; Log-rank test, df = 1, $\chi^2 = 0.180$, p = 0.671; Fig. 3.18-B). A power analysis on males indicated a necessary sample size of 1623 flies to detect a statistically significant effect.
Figure 3.18 – Effects of yeast on starvation resistance in D. melanogaster. Even though there was not a statistically significant difference between treatments, female flies (A) seemed to survive longer when fed live L. kluyveri 48 h before starvation started (n = 10 flies/treatment; Log-rank test, df = 1, $\chi^2 = 0.637$, p = 0.425). Survival of males (B) was not affected by the yeast provided 48 h before starvation (n = 10 flies/treatment; Log-rank test, df = 1, $\chi^2 = 0.180$, p = 0.671).
4 Discussion

Yeasts are an important part of *Drosophila* diet because of their nutritional value, but as living microorganisms, they also have the potential to interact with the fly as their host. The gut microbiota affects many aspects of *D. melanogaster* physiology, including development (Storelli et al., 2011), metabolic functions (Wong et al., 2014), immune responses (Glittenberg et al., 2011), and mating success (Sharon et al., 2011). Yeasts affect fly physiology, but the effects have previously been ascribed to nutritional properties, rather than their being members of the resident gut microbiota. The overall goal of my project was to determine if yeasts are part of the gut microbiota and, as such, could affect *D. melanogaster* physiology. Here I show that some yeasts affect fly physiology: they reduce development time and chill coma recovery time; and that yeasts need to be alive to induce a strong effect, implying a biological interaction between yeast and *Drosophila*.

4.1 Yeasts as members of the *Drosophila* gut microbiota

The *Drosophila* gut microbiota is constantly changing in number and species of microbes depending on the microorganisms found in the food they consume (Erkosar & Leulier, 2014). Diet and habitat dictate the microbial species, while the constant inoculation through ingestion is necessary to maintain colonization (Chandler et al., 2011; Blum et al., 2013). Even under these fluctuating conditions, bacteria associated with the *Drosophila* gut affect the physiology of the host (Broderick & Lemaitre, 2012). In a similar manner, yeast species in the fly gut vary depending on the diet, and the number of live yeast cells in the gut declines with time (Lachance et al., 1995; Chandler et al., 2012; Hoang et al., 2015). While the consensus is that bacteria are part of the gut microbiota, yeasts have been only explored in the context of their nutritional value (Vega & Dowd, 2005). Early recognition of the nutritional value of yeasts and dead yeasts meeting the nutritional requirements of most *Drosophila* species, as well as the low number of yeast cells surviving passage through the flies’ gut when compared to bacteria, likely contribute to the underestimation of the importance of yeasts as gut microbes (Broderick & Lemaitre, 2012). However, I demonstrated that yeasts persist in the gut of
the fly, and this persistence is species-specific. In addition, I have shown that yeast effects on fly CCRT and development time are dependent on the yeast being alive.

In nature, many *Drosophila* species consume yeasts on a regular basis (Phaff *et al*., 1956), and here I present evidence of this being a mechanism for maintaining the population of live yeast in the gut of the fly. Through phase-contrast microscopy I demonstrated that while yeasts begin forming spores in the crop of the fly, many cells remain in their vegetative state maintaining intact cell walls (Fig. 3.5). I found that ascospores are more abundant in the hindgut of the fly, with most other yeast cells appearing to have undergone digestion (Fig. 3.6-A; also Coluccio *et al*., 2008). However, I did observe a small number of vegetative cells in the frass, some of which were reproducing (Fig. 3.6-B). The presence of high numbers of live yeast in the crop combined with those able to survive passage though the fly’s digestive tract (even if in low numbers), suggests that the contribution of yeasts to their host is not solely due to the digestion of yeast. Live yeast in the crop may be affecting fly physiology by actively releasing nutrients or modifying food before it passes to the midgut. Alternatively, molecules released by the yeast might affect *D. melanogaster* signaling pathways directly within the crop epithelium or further down the digestive tract.

### 4.1.1 Factors affecting yeast persistence in *Drosophila melanogaster* gut

I found that persistence of yeast in the gut of *D. melanogaster* varies among species of yeast. Most of the early yeast persistence experiments used *S. cerevisiae* in the form of commercial baker’s yeast (El-Tabey Awab Shihata & Mrak, 1951). *S. cerevisiae* is not one of the species of yeasts commonly recovered from the gut of wild-caught *Drosophila* (Phaff *et al*., 1956) and, as I demonstrated, has a short persistence time when compared to *L. kluyveri*, which can be found in the gut of some *Drosophila* species in nature. I found that *L. kluyveri* can be cultured from the gut of *D. melanogaster* for up to five days while *S. cerevisiae* is completely cleared by the second day (Fig. 3.1). El-Tabey Awab Shihata and Mrak (1951) suggested that yeast (*S. cerevisiae*) only persists for 24 h in the fly because it gets digested after leaving the crop. However, yeasts originally isolated from various *Drosophila* spp. have different persistence times (Hoang *et al*., 2015). Since
persistence is species specific, some species of yeasts are more likely to become gut residents and modify fly phenotype by interacting with the host’s physiology.

A difference in the ability of *L. kluyveri* and *S. cerevisiae* to persist in the *Drosophila* gut may be the result of the yeast tolerance to the gut environment (Hoang *et al.*, 2015) or the immune system of the fly recognizing some yeasts as symbionts in the same way it recognizes bacteria (Feldhaar & Gross, 2008). The gut epithelium of *D. melanogaster* produces reactive oxygen species (ROS) in response to live yeast (Ha *et al.*, 2009); yeast species able to tolerate ROS are more likely to have longer persistence. Ha *et al.* (2009) only used baker’s yeast, but Hoang *et al.* (2015) demonstrated that *Hanseniaspora occidentalis* is able to grow under high ROS conditions (a solution of *H*₂*O*₂), and that it persists longer than *S. cerevisiae* in the gut of *D. melanogaster*. Alternatively, yeast cells may form biofilms to anchor themselves to the gut epithelia of the fly in the same manner as bacteria in the gut of bean bugs (Kim *et al.*, 2014) and the opportunistic pathogenic yeast, *Candida albicans*, in the gut of humans (Mathé & Van Dijck, 2013). In future, the potential for *L. kluyveri* to colonize the gut in a formal sense could be tested by exploring its ability to grow under high ROS (Hoang *et al* 2015), and its ability to form biofilms (Reynolds & Fink, 2001).

The methods most commonly used to determine persistence of yeast in the *Drosophila* gut might overestimate persistence time since there is no way to avoid the constant inoculation of the food by the flies (and subsequent re-ingestion of yeast cells). However, I periodically transferred flies to sterile food every 24 h minimizing the re-ingestion of high numbers of yeast cells by the flies. Serial transfers to sterile media reduce the amount of microbes – both yeast and bacteria – available to the flies (Blum *et al.*, 2013; Hoang *et al.*, 2015). Conducting the serial transfers even more frequently can further minimize the number of yeasts available to the flies within the ‘sterile’ food vials (Hoang *et al.*, 2015). Alternatively, individual flies can be immobilized to avoid re-inoculation of food, with the yeast and sterile food presented directly to the fly’s proboscis such as in proboscis extension reflex assays (Bakula, 1969; Shiraiwa & Carlson, 2007).
4.1.2 Between and within sex variation in *Drosophila melanogaster* gut-yeast

In *Drosophila*, the bacterial gut microbiota composition varies among individuals of the same species, even when reared on the same food source (Wong *et al.*, 2014). Similar variability might exist in the yeasts found within the gut of flies, but since the guts have usually been pooled during testing, interindividual variation has not been explored (Starmer & Lachance, 2011; Chandler *et al.*, 2012). During the persistence experiments, I dissected and homogenized the guts of single individuals; I found that the number of colonies grown in YM agar plates varied greatly among flies, even those kept in the same vial. Since the *D. melanogaster* population I used in these experiments was not isogenic, genetic variation among individuals could have resulted in the high variation in yeast colony numbers in the same way that genetic polymorphism affects gut microbiota in mice (Kovacs *et al.*, 2011). In general, female flies had significantly more yeast cells in their gut than males. Variation between males and females is at least partially explained by the amount of yeast the flies consumed. While female flies are immediately attracted to yeast and ingest large amounts, males do not seem as attracted to yeast and consume only minimal quantities (Fig. 3.4–3.6). In vertebrates, sex influences gut microbiota composition (Markle *et al.*, 2013) and this could be the result of individual diet (Bolnick *et al.*, 2014). In *Drosophila*, sex differences in yeast consumption and the resulting colonization of the gut by the yeast may be driven by the higher energy requirement of females during egg production (Parisi & Oliver, 2011). Sex and genotype should thus be considered when studying the microbial gut composition and function of animals in nature.

4.2 Effect of yeasts on *Drosophila melanogaster* physiology

Yeasts are important for *D. melanogaster* fitness; they reduce development time (Anagnostou *et al.*, 2010), increase fecundity and longevity (Skorupa *et al.*, 2008), and promote immunological responses against pathogens (Vass & Nappi, 1998). However, the yeast responsible for these effects on fly physiology was *S. cerevisiae* obtained from commercial baker’s yeast, with the exception of Anagnostou *et al.* (2010) who also used
*Kluyveromyces lactis*, *Metschnikowia pulcherrima* and *Pichia toletana* in their treatments. Aside from Skorupa *et al.* (2008), all of these studies used live yeast, yet all regarded them solely as dietary yeasts and not symbionts. While some of these effects are the result of the nutritional value of the yeast, effects due to the yeast being alive and interacting with the host were not considered. Here I show that while development time was slightly affected by dietary yeast (heat-killed yeast), the reduction of development time in flies reared in vials inoculated with live yeast was far more significant. Furthermore, reduction in CCRT was only present in females fed with live *L. kluyveri* 48 h prior to chill coma induction. Therefore, I conclude that while the dietary value of yeast may be important in development, the biological interaction between live *L. kluyveri* and *D. melanogaster* is necessary to affect CCRT in *D. melanogaster*.

4.2.1 Live yeasts decrease *Drosophila melanogaster* development time

Development time is important because it is a life history trait based on genetic makeup, and can modify overall fitness in *D. melanogaster* by affecting generation time, exposure to competition at high densities, and intrinsic rate of population increase (Kohane, 1994; Yadav & Singh, 2005). I found that *D. melanogaster* reared in vials inoculated with live yeast, either *S. cerevisiae* or *L. kluyveri*, eclosed up to 40 h before flies in the axenic treatment, with 90% of the gnotobiotic flies reaching adulthood before the first axenic flies emerged from their pupae. These results indicate that live yeasts play an important role in the development rate of *D. melanogaster* and that *S. cerevisiae* is as beneficial for development as *L. kluyveri*. In nature, most *Drosophila* larvae develop in substrates containing different species of yeast (Carson *et al.*, 1956); here I show that while some phenotypes may suffer from the lack of natural gut-associated yeasts in laboratory populations that only have access to *S. cerevisiae*, development time is not one of them.

I showed that live yeasts reduce development time in *D. melanogaster* (in accordance with Anagnostou *et al.*, 2010); therefore, I tested whether the effect of yeasts on development time was the result of dietary yeast (heat-killed) or live yeast acting as a symbiont. I found that although live yeast reduced development time by 40 h, high
concentrations of heat-killed yeasts (2× and 8× the original live *S. cerevisiae* and *L. kluyveri* respectively), only reduced development time by 15 or 7 h respectively. Heating yeasts suspensions at 60 °C effectively kills the yeast cells and increases the availability of nitrogen and protein for animal consumption (Mogren *et al.*, 1973). However, many molecules such as enzymes and small proteins undergo unfolding and inactivation at temperatures above 55 °C (Wang *et al.*, 2004). Thermal denaturation of micronutrients may affect the nutritional value of the heat-killed yeasts. Therefore, I conclude that while the contribution of the yeasts to reducing development time may be the result of a complex biological interaction between the yeasts and *D. melanogaster*, nutritional factors cannot be ruled out. To investigate this further, nutritional analysis for live and heat-killed yeast should be done.

Differentiating the nutritional benefits of dietary yeasts from those obtained through symbiotic interactions between the yeasts and their host presents methodological challenges. Even though I used heat-killed yeasts at eight times the concentration of live yeasts, live yeasts kept replicating within the vials where the flies were reared. Therefore, the larvae had a constant supply of live yeast throughout their development while larvae in the heat-killed yeast treatments may only have had access to the yeast during early development. Furthermore, I inoculated the food vials by pipetting the various yeast solutions on the surface of the media and therefore the yeasts were not necessarily available to the larvae feeding deeper within the food (Durisko *et al.*, 2014). One way to increase the accessibility of dietary yeast to burrowing larvae would be to include higher concentrations of heat-killed yeast in the food so that the larvae have a constant supply of dietary yeast within the medium (Wong *et al.*, 2014).

In *Drosophila melanogaster*, the bacterial gut microbiota influences fly metabolism, affecting body size and development time in a sex dependent manner (Wong *et al.*, 2014). Yeasts may also affect fly performance through changes in the host’s metabolic responses. Eliminating key nutritional components or altering the *Drosophila* diets through the use of supplements and then comparing development time and body size of axenic flies and gnotobiotic flies (those with live yeasts in their gut) could help determine the contribution of gut yeasts to development.
4.2.2 Live yeasts reduce chill coma recovery time in *Drosophila melanogaster*

Chill coma recovery time is commonly measured as a proxy for low temperature tolerance in *Drosophila* spp. (Andersen *et al*., 2015). I found that live *L. kluyveri* reduces chill coma recovery time by up to 10 min in females only (Fig. 3.10). Adult flies were fed with live yeasts for only 48 h, and therefore any phenotype modification on *Drosophila* had to have happened within that narrow time frame. This suggests that the yeast gut microbiota may be a driver of phenotypic plasticity in *Drosophila* cold tolerance (Lee & Denlinger, 1991) and that this effect is sex dependent. Thermal tolerance can be used as a predictor for *Drosophila* geographical limits, but since males’ CCRT was not modified by yeast because they do not ingest it, the interaction between these two observations likely determines the actual population geographic range. However, it is possible that *Drosophila* males will increase their yeast consumption at low temperatures therefore obtaining the benefits of yeasts in their gut.

Repeated *in vitro* subculturing of yeasts modifies their original characteristics, such as spore formation and properties of spore surfaces (Shah *et al*., 2007; Nahar *et al*., 2008). I kept a culture of *L. kluyveri* on YM agar for over a year through subculturing techniques at 25 °C. When I used this culture to test CCRT, I observed that female flies which responded previously to the yeast by recovering from chill coma earlier than their axenic counterparts were no longer responding to the treatment. *L. kluyveri* spores start forming in the crop of the fly and spores are the yeast structure that most routinely survives passage through the *Drosophila* gut. I suggest that this characteristic of the *L. kluyveri* that was lost through repeated sub-culturing was important for the effect of the yeast on CCRT. Subsequently, I obtained a fresh culture of *L. kluyveri* (same strain, NRRL Y-12651) from UWOPS. Female *D. melanogaster* fed live yeast from this fresh culture recovered 5 min faster from chill coma than axenic females. Therefore, sporulation may be of importance in CCRT. This need for sporulation could be a useful tool for exploring the mechanisms underlying the yeast-fly interaction, and may also explain why not all yeast experiments yield a phenotype (Anagnostou *et al*., 2010).
Since I observed flies recovered in groups of five and selected only one fly per group for dissection and plating of the gut, I was not able to discern a pattern between time of recovery and amount of yeast in the gut for most experiments. However, I conducted a separate CCRT experiment measuring the time of recovery and number of yeast colony forming units in individual flies. I found there is a correlation between the number of CFU and the time at which the flies recovered from chill coma. This implies that interindividual variation in CCRT could be driven by variation in the amount of yeast in the \textit{D. melanogaster} gut. Different \textit{Drosophila} species recover from chill coma at different times (Overgaard \textit{et al.}, 2011), as do populations of the same species found at different latitudes (Ayrinhac \textit{et al.}, 2004). In addition, acclimation at low temperature reduces CCRT in \textit{D. melanogaster} (Ransberry \textit{et al.}, 2011). However, it remains unknown how much of these differences are the result of the interaction between the \textit{Drosophila} and its microbiota. Here I show that live yeast in the gut reduces CCRT in \textit{D. melanogaster} and that the amount of yeast recovered from individual flies correlates with their recovery time. Therefore, the presence or absence of yeast in the \textit{Drosophila} gut may prove to be a powerful tool for exploring the mechanisms responsible for the variation in CCRT between \textit{Drosophila} species and their different populations.

I tested CCRT in \textit{D. melanogaster} fed with different concentrations of heat-killed yeast to identify if the decrease on recovery time was the result of the nutritional value of the yeast. As with the live yeast treatments, I observed that males fed on live yeast recovered at the same rate as axenic ones and those fed on different concentrations of heat-killed \textit{L. kluyveri} (Fig. 3.8 B). Females in the live \textit{L. kluyveri} treatment recovered 10 min faster than those in the axenic or heat-killed treatments. The CCRT of females fed high concentrations of heat-killed yeast did not differ from the flies in the axenic treatment. Furthermore, the subcultured yeast did not have an effect on CCRT but should have provided the same nutrients as the fresh yeast culture. The lack of effects on CCRT in males is not surprising since adult males do not consume yeast in large quantities. In females however, these results indicate that live yeasts in the crop or the live yeast passing through the gut might be providing protection against cold stressors.
I propose that CCRT is a valuable assay for exploring the effects of gut-associated yeasts on *Drosophila* physiology and the mechanisms that are responsible for these effects. Measuring CCRT is easy, does not require intensive training or expensive equipment (David *et al*., 1998). Furthermore, since adult flies are allowed to feed on live yeasts for only 48 h, other confounding effects that happen through development are avoided. During chill coma, water and ion homeostasis is lost and the recovery of this homeostasis is necessary for the fly to regain movement (MacMillan *et al*., 2012). The process by which osmotic balance is re-established is energetically costly (MacMillan *et al*., 2012). Since live *L. kluyveri* reduces CCRT, it is possible that the live yeast in the crop and those that survive passage though the gut produce osmolytes that aid in the recovery of ion and water balance. Alternatively, the live *L. kluyveri* in the *Drosophila* gut may increase the ability of the fly to sustain an elevated metabolic rate during the recovery from chill coma.

Gut-associated yeasts may affect CCRT through large-scale changes in *D. melanogaster* physiology. Transcriptomic analyses have revealed that the bacterial gut microbiota affects *Drosophila* gene expression; Broderick *et al*. (2014) identified 121 upregulated and 31 downregulated genes in *D. melanogaster* infected with bacteria isolated from the gut of conventionally reared flies. The majority of the modified genes affect immune responses, metabolism, gut morphology, and tissue homeostasis (Broderick *et al*., 2014). Furthermore, they found that 53 % of the upregulated genes were affected through the immune deficiency (Imd) pathway by comparing the change of gene expression between wild type *D. melanogaster* with a Imd-deficient mutant (Broderick *et al*., 2014). In a similar study, Erkosar *et al*. (2014) identified 105 transcripts that were upregulated in the midgut of *D. melanogaster*. Similar methodology can be applied to detect changes in gene expression due to the presence of live yeast in the *Drosophila* gut and different mutant lines can be used to identify possible pathways responsible for these changes.

4.3 Implications and future directions

The gut microbiota of *Drosophila*, even though taxonomically less complex than that of vertebrates, is composed of various species of bacteria and yeasts (Chandler *et al*.,
Most studies have focused on the role of gut bacteria in *Drosophila* physiology, while the role of gut-associated yeasts remains largely ignored (Chandler *et al.*, 2012). Bacteria in the microbiota affect *D. melanogaster* physiology (Shin *et al.*, 2011; Storelli *et al.*, 2011), yet many studies have used fecal matter from conventionally reared *Drosophila* to replenish the gut microbiota of axenic flies without determining microbial composition (Glittenberg *et al.*, 2011; Buchon *et al.*, 2009; Wong *et al.*, 2014). I have demonstrated that yeasts have the potential to become residents of the gut microbiota and that they affect *D. melanogaster* physiology as well. Treating yeast solely as dietary supplements ignores the role of yeasts as gut microbes and the possible effects that these can have on fly physiology, including the interaction with bacteria. Since yeasts are found in the gut of animals, live yeasts can be transmitted to test animals by the introduction of fecal matter from their conventionally reared counterparts (Broderick & Lemaitre, 2012). The function and mechanism by which gut-yeasts affect not just *Drosophila* physiology but the physiology of other insects need to be further explored.

*Drosophila* spp. in nature encounter various species of yeast, but *S. cerevisiae* (particularly the strains used in commercial active yeast) is not one of the species commonly isolated from the gut of wild-caught flies (Chandler *et al.*, 2012). Yet in laboratory-reared *Drosophila* populations, *S. cerevisiae* is the yeast of choice because it is commercially available in large quantities and seems to provide the necessary nutrients for normal development (Broderick & Lemaitre, 2012). However, I demonstrated that *L. kluyveri* persists longer than *S. cerevisiae* indicating that some species of yeast are more likely to become members of the gut microbiota. Also, *L. kluyveri* reduces *Drosophila* CCRT but only when the yeast is alive, adding further evidence that it is a functioning member of the gut microbiota. Therefore, other yeasts species – especially those isolated from *Drosophila* previously – should be incorporated into the study of the effects of gut microbes on *Drosophila* performance.

4.4 Conclusions

Yeasts are constantly associated with *Drosophila* and the presence or absence of live yeast affects fly physiology, suggesting that yeasts form part of the resident gut microbiota. I have demonstrated that a *L. kluyveri* is able to survive in the crop and
effectively colonize it by the fly constantly ingesting yeast cells. Furthermore, yeast persistence in the *Drosophila* gut is yeast species-specific. Since chill coma recovery time is reduced by *L. kluyveri* and the effect is only present when the yeast is alive, I conclude that this system: *D. melanogaster, L. kluyveri* and CCRT is a good model for exploring the mechanisms by which yeasts influence host physiology.
References


Appendix A – Instructions for growing axenic *Drosophila*

Since *Drosophila* embryos do not acquire their microbiota until they emerge from their eggs, surface sterilization of the eggs and growth under sterile conditions allows for the development of axenic (microbe-free) adult flies.

**Materials**

- Laminar flow cabinet
- Bacti-Cinerator IV (McCormick Scientific)
- Aluminum foil
- Absorbent paper towel
- Nylon filters (⌀ = 24 mm, NY2002500, EMD Millipore)
- Metal inoculation loop
- Micro spatula (20 cm)
- Smooth tip forceps (#3)
- 1000 µL pipette and pipette tips
- 70 % ethanol (for sterilizing tools and eggs)
- Deionized Water (dH$_2$O)
- Sterile Phosphate buffer saline (PBS tablets, Sigma-Aldrich, P4417). Prepare the PBS buffer by dissolving a tablet in 200 µL dH$_2$O as per manufacture’s instruction. Create aliquots (3 mL) in test tubes and autoclave (121 ºC for 15 min).
- Sterile Petri dish with a thin layer of media (1.5 g agar, 1.5 g active yeast, 4.3 g sugar, 100 mL dH$_2$O) to receive the sterilize eggs. Mix all ingredients in a Erlenmeyer flask and autoclave (121 ºC for 15 min) before pouring a thin layer on sterile Petri dishes (⌀ = 100 mm). Let it cool down for one hour.
- Active yeast paste (Fleischmann’s Yeast, Farinex, QC, Canada). Rehydrate the yeast with a small volume of water until it forms a paste with the consistency of smooth peanut butter.
- Autoclaved 35 mL glass vials containing 10 mL autoclaved Tucson food (1 L dH$_2$O, 45 g sugar, 30 g cornmeal, 18 g active yeast, 12 g agar; adapted from Markow & O’Grady, 2006). Let the vials sit at room temperature overnight.

Adult flies are kept in small acrylic egg collection cages ($\varnothing = 30.5$ mm, $h = 50.8$ mm), with petri dishes ($\varnothing = 30.5$ mm) filled with grape/apple agar (100 mL fruit juice, 100 mL dH$_2$O, 3 g agar; adapted from Merkling & Rij, 2015) topped with active yeast paste (Fleischmann’s Yeast, Farinex, QC, Canada) to stimulate oviposition. Replace the agar plates for new ones without yeast paste; these are plates from which eggs are going to be collected. Allow the flies to oviposit for a minimum of 3 h depending on the number of females in the cages. The sterilization procedure is more effective when washing 50-200 eggs at the time.

**Instructions**

1. Prepare as many aluminum packages as needed (one per plate): cut an aluminum foil rectangle (30 cm $\times$ 24 cm), fold the paper towel (28 cm $\times$ 8 cm) in half and place it at the centre of the aluminum foil. Put one of the filters inside the folded paper and fold the edges of the aluminum foil around the paper to fully cover it. This is then ready for autoclaving (121 ºC for 15 min; Fig. A.1).

2. Sterilize all surfaces in the laminar flow cabinet and place all necessary materials inside.

3. Sterilize the loop, forceps and spatula with the Bacti-Cinerator, holding them inside until the alcohol on the tip evaporates (5 to 7 seconds).

4. Extend an aluminum package, taking care not to touch the napkin or the filter with other than the sterilized tools. Move the filter to the left with the forceps (ready to receive the eggs) (Fig. A.1-B).

5. Place the sterilized end of both tools on the package. This will keep the tools clean and will also hold the paper and aluminum in place.

6. Get one of the plates with eggs. Cover the eggs in the plate completely with 70% ethanol (≈ 600 µL).
7. Separate the eggs from the agar by carefully moving them with the loop. Keep moving the eggs in the ethanol for 5 min. Add more ethanol if necessary to keep the eggs completely submerged.

8. Tilt the plate on an angle to help collect the eggs on one side and pour the alcohol and eggs onto the filter. Let the paper absorb the alcohol.

9. Move the filter with the eggs to the right and rinse with PBS (~ 600 µL) using the pipette. Do this two more times (move the filter to a dry spot and rinse) (Fig. A.1-C).

10. Lift up the filter and place it upside down on the fly food plate, making sure the eggs are against the food.

11. Repeat steps 3-10 for the remaining plates.

12. Sterilize the spatula. Wait for it to cool down and then gently use it to apply pressure to each filter. This makes the eggs stick to the food so as to not remove them along with the filter.

13. Sterilize the forceps and remove the filter from the food plate by holding one edge of the filter and peeling it off. The filters can be washed and reused.

14. Sterilize the spatula and use it to divide each area with eggs (where the filter used to be) into small squares containing approximately 50 eggs each.

15. Bring the glass vials into the laminar flow cabinet. You no longer need the pipette, pipette tips, and PBS. Remove these from the cabinet to make room for the vials.

16. Sterilize the narrow end of the spatula. Cool it down by touching the food where there are no eggs. Pick up one of the small squares with the spatula and put it in the vial (with the eggs facing up). Plug the vial and incubate undisturbed at standard conditions (21.5 ± 1 °C, 60 ± 5 % relative humidity, 13 h:11 h L:D).
Figure A. 1 – Aluminum foil package for *Drosophila* eggs sterilization

A: Aluminum package

B: Filter ready to receive *Drosophila* eggs

C: Filter moved for egg rinsing
## Curriculum Vitae

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