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Ferulic Acid Produced by *Lactobacillus fermentum* NCIMB 5221 Reduces Symptoms of Metabolic Syndrome in *Drosophila melanogaster*

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Abstract

The gut microbiota is an intricate community of thousands of bacterial species living in the gastrointestinal tract critical for vitamin synthesis, mineral absorption, digestion of otherwise indigestible fibers and the extraction of energy from food. Recently, the health of the gut microbiota's community architecture has become linked to energy-regulatory diseases including metabolic syndrome: a collection of symptoms including hyperglycemia, hypercholesterolemia, high blood pressure, increased abdominal fat and triglyceride levels. However, the mechanism of communication between the gut microbiota and the host energy metabolism remains elusive. The current study shows that ferulic acid (FA) produced by the intrinsic ferulic acid esterase activity of the probiotic bacteria *Lactobacillus fermentum* NCIMB 5221 (Lf5221) can dose-dependently rescue the phenotypic markers of diet-induced diabetes and obesity in *Drosophila melanogaster*. In Drosophila exposed to either a high-sugar or high-fat diet, living Lf5221 at 2.5 or 7.5×10^9 CFU/ml media effectively rescued whole-body weight, glucose, trehalose and triglyceride levels. All of the aforementioned effects were lost in heat-inactivated bacteria indicating that a metabolic product is responsible. Likewise, FA at 0.5 mM in the metabolically challenged Drosophila models reared similar effects on the physiological markers while also reducing hyperglycemia in the circulating hemolymph. On the signaling level, the high-sugar diet predictably had an elevated expression of the Drosophila insulin-like peptides 2, 3 and 5 and in the high-fat diet, an increase in fatty acid synthase, acetyl-CoA carboxylase and phosphoenolpyruvate carboxykinase expression. On both diets, Lf5221 and FA rescued gene expression, at different concentrations, to the level of controls. Examining the mechanistic gene expression, both Lf5221 and FA rescued expression of dFOXO and dTOR, but not dAkt indicating that FA produced by Lf5221 is acting on one of the downstream-signaling molecules from the insulin receptor, possibly dTOR: an overall energy regulator in flies and humans alike. The present study for the first time outlines a streamlined mechanism for how the gut microbiota communicates with the host's energy-regulating metabolism. Proper supplementation with ferulic acid esterase active probiotics such as Lf5221 could potentially prevent or alleviate symptoms of metabolic syndrome and other energy-regulating diseases including diabetes, obesity and neurodegeneration.

Abbreviations

ACC: Acetyl-CoA Carboxylase; CFU:Colony Forming Units; dAkt: Drosophila Protein Kinase B; *dilp: Drosophila insulin-like peptide*; dFOXO: Drosophila Forkhead Box Protein O; dTOR: Drosophila Target of Rapamycin; FA: Ferulic Acid; FAE: Ferulic Acid Esterase; FAS: Fatty Acid Synthase; GIT: Gastrointestinal Tract; HFD: High Fat Diet; HSD: High Sugar Diet; IlS: Insulin-like Signaling; Lf5221: *Lactobacillus fermentum* NCIMB 5221; MRS: Man-Rogosa-Sharpe; PEPCK: Phosphoenolpyruvate Carboxykinase; Upd2: Unpaired 2

Introduction

The gut microbiota is constituted by a plethora of bacterial species residing symbiotically in the gastrointestinal tract (GIT). These estimated 10^{13}-10^{14} bacterial cells are indispensable to the host's wellbeing as they form a dense synergistic ecosystem that impacts host digestion of vitamins, minerals and otherwise indigestible fibers, extraction of energy from foods, production of signaling molecules and hormones and modulation of gastrointestinal dynamics including motility and transit time [1]. The microbiota may be considered as an organ onto itself, being responsible for a variety of physiological activities including metabolism, neurological development, energy homeostasis, immune regulation and fundamental signaling cascades.

Recently, the health of the gut microbiota has been linked to the pathology of metabolic syndrome, a growing health and economic concern. Metabolic syndrome is a collection of energy-regulating risk factors including hyperglycemia, pre-diabetes, abdominal obesity, elevated cholesterol and triglycerides and high blood pressure leading to an elevated risk of cardiovascular disease [2]. In the United States alone, there was an increased prevalence from 32.9 to 34.7% diagnosed cases, mostly in women and persons over the age of 60 years [3]. Although risk factors vary based on demographic and gender differences [4], the increasing global sedentary lifestyle and proportionally aging population has made the incidence of metabolic syndrome rise to epidemic proportions [5].

It has been recently recognized that the gut microbiota is pinnacle for regulating metabolic energy homeostasis, especially in the context of metabolic syndrome [6,7]. Depending on an individual's diet, the gut

microbiota will adapt to the nutritional phenotype and be composed of different proportions of beneficial and pathogenic species. For example, on a typical western diet laden with refined sugar and fats, there will be an over representation of the phyla Firmicutes and an under representation of the phyla Bacteriodetes. An increase in this ratio is linked to adiposity, hypertriglyceridemia, diabetes and increased lipopolysaccharide (originating from gram-negative bacteria) levels that contribute to systemic inflammation [8].

The role of gut microbiota in metabolic syndrome was first demonstrated by Backhed et al. using germ-free rodents, animals free of all microorganisms both in and on them. When the microbiota of conventionally raised animals was transferred to germ-free rodents, the latter demonstrated a 60% increase in adiposity and insulin resistance, regardless of decreased muscle mass and food intake [9]. Similarly, Ley et al. demonstrated that ob/ob mice, a genetic model of obesity due to a mutation in the leptin gene, have a distinct microbiota when compared to their lean counterparts. Specifically, the obese mice demonstrated a 50% reduction in Bacteroidetes (Gram-negative) and a 50% increase in Firmicutes (Gram-positive) counts [10]. Interestingly, in a very recent study, it was shown using the gut microbiota from mouse twins discordant for obesity, that there is a strong interaction between the microbiota and diet [11].

Certain probiotic bacterial strains have demonstrated important anti-inflammatory potential, both *in vitro* [12,13] and *in vivo* [14], which is beneficial for the management of chronic systemic low-grade inflammation present in metabolic syndrome. Probiotic strains have also demonstrated the capability to lower cholesterol levels, linked to atherogenic dyslipidemia [15]. In addition, specific probiotic strains have been shown to impact the development of obesity in humans [16]. In diabetes, there is a characteristic reduction of Firmicutes and other bacterial species including, *Bacteroides vulgatus, Faecalibacterium prauznitzii* and the Bifidobacterium, Lactobacillus and Roseburia genus [3]. This creates an un-healthy GIT environment that is more prone to the growth of opportunistic pathogens and pro-inflammatory species that aggravate metabolic phenotypes. In addition, an increase in fecal levels of *Lactobacillus gasseri, Streptococcus mutans* and *Escherichia coli* is predictive of insulin resistance. When insulin-resistant males received lean donor fecal transplantations, there was a significant increase in intestinal microbial diversity and a distinct increase in butyrate-producing bacteria such as *Roseburia* and *Faecalibcaterium* spp. in the feces and *Eubacterium halii* in the small intestine which improves GIT health and alleviates the metabolic symptoms.

With the microbiota's importance in health and disease, it has become a therapeutic target [17]. There are several ways that the gut microbiota can be altered including the consumption of health-promoting bacterial probiotic species, indigestible fibers or prebiotics and a combination of the two known as synbiotics. The Food and Agriculture Organization of the United Nations and the WHO define probiotic bacteria as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Several probiotics have been identified to be beneficial to the metabolic syndrome due to their insulin-regulating, anti-obesity and anti-inflammatory effects. Many probiotic species secrete metabolically active products or digest otherwise indigestible fibers (prebiotics) to produce signaling molecules with downstream metabolically active effects. These include the short-chain fatty acids (SCFAs), that regulate energy metabolism (reviewed in [18]), but there are many other products that also elicit beneficial effects.

In the present study, the ferulic acid esterase (FAE)-active probiotic *L. fermentum* NCIMB 5221 (herein referred to as Lf5221) reduced a variety of metabolic stress markers induced by a high-sugar or a high-fat diet in *Drosophila melanogaster*. Ferulic acid (FA, 4-hydroxy-3-methoxy cinnamic acid) is released from plant polymers, mostly lignans and other esterified substances by the FAE activity of bacteria [19]. FA boasts many beneficial properties including reducing insulin resistance, triglycerides, cholesterol blood pressure, chronic inflammation, oxidative stress and more physiological manifestations of metabolic distress (rev. in [20]. It is in this context that the specific physiological and molecular effects of Lf5221-generated FA on metabolically challenged *Drosophila melanogaster* was investigated in order to delineate a molecular mechanism of communication from probiotic therapy and changes in the gut microbiota with metabolic management of host physiology.

Materials and Methods

Bacterial strain and culture conditions

L. fermentum NCIMB 5221 (Lf5221) was obtained from the NCIMB Culture Collection (Aberdeen, Scotland, UK). Cells were cultured with Man-Rogosa-Sharpe (MRS) media obtained from Sigma Aldrich (Oakville, ON, Canada) at 37°C on MRS-agar plates or in liquid media. After one round of liquid culture, several bacterial stocks were made in MRS containing 20% (*v/v*) glycerol and stored at -80°C. As constant culturing was required to carry out all experiments, bacterial stocks were renewed from the frozen stock bi-weekly in order to maintain culture purity. To preform each individual experiment, a 1% (*v/v*) inoculum was used for subculturing, incubated at 37°C for 18 h and removed immediately before use. To prepare the individual Drosophila bottles, the overnight culture was centrifuged at 4000 rpm for 10 min at 4°C. The pellet was washed once and resuspended in 0.85% (*w/v*) physiological saline. Total colony counts were determined by spectrophotometry compared to a standard curve prepared with colony forming units (CFUs) on MRS plates. Heat inactivated bacteria were prepared by heating the concentrated bacterial solution at 95°C for 20 min. This method was verified by plating heat-treated bacterial cells on MRS-agar plates at various dilutions and counting any possible CFUs. With this treatment regime, there were no CFUs at any dilution after 72 h of incubation at 37°C.

Drosophila melanagaster strains and media

Wild type *Drosophila melanogaster* (Oregon R) were procured from the Bloomington Drosophila Stock Center (Indiana University, Bloomington Indiana). Flies were reared on a standard cornmeal-sucrose-yeast media without active yeast culture prepared by boiling the cornmeal (83 g), sucrose (50 g) and yeast extract (30 g) in distilled water for 30 min. Drosophila were kept in controlled conditions with a 12 h:12 h light-dark cycle at 25°C. Baseline media contained 0.15 M sucrose whereas the high-sugar diet (HSD) contained 1.0 M sucrose and the high-fat diet (HFD) contained an additional 20% (*v/v*) coconut oil. Diabetes or obesity was induced by transferring 7 day old flies reared and kept on normal probiotic-containing media to a HSD or HFD, respectively also containing the probiotic for 21 days at which point the flies were sampled. Inoculated media was prepared by partitioning the concentrated bacterial culture into the cooled, yet liquid, media to final concentrations of 5.0×10^8 to 7.5×10^9 CFU/ml media. This is a verified method of oral-inoculation to flies and it was verified that bacterial cells remained viable in the Drosophila media for up to two weeks before a detectable loss of concentration by daily CFU counting. Nevertheless, flies were transferred to new inoculated bottles every 3-4 days during the course of an experiment.

Drosophila flies sampling

After three weeks of treatment with Lf5221, FA and/or HSD/HFD flies were anesthetized in an ether chamber at 10 am, 2 h after lights-on. Hemolymph was collected by piercing the Drosophila's thorax with a thin needle and collecting 40 flies in a small tube with several holes situated in a larger centrifuge tube. Samples were centrifuged at 5000 rpm for 5 min at 4°C to collect hemolymph. Samples were immediately placed on ice and all subsequent experiments were conducted immediately after collection. Whole-body homogenates were prepared by homogenizing on ice 20 flies in 200 μl of 10 mM Tris-EDTA, 0.5% Triton X-100 buffer, pH 7.4. Samples were gently centrifuged for 3 min at 1000 rpm and 4°C to remove debris.

Measure of self-reproducibility

To measure the self-reproducibility of Lf5221 in Drosophila after oral inoculation, two-day-old flies were starved in empty vials for 3 h (to enable synchronous feeding) prior to being transferred to fresh vials containing various concentrations of Lf5221. After 24 h of inoculation, flies were surface sterilized in 10% bleach, rinsed thrice in distilled water and transferred to sterile (autoclaved) media in a sterile hood. The removal of all surface bacteria and feces was verified by plating the wash water on MRS-agar plates to ensure no CFUs formed within 72 h. The surface sterilization and transfer to sterile media was repeated daily to limit cross-contamination of feces to flies. Samples of flies were taken daily and assessed for the presence of Lf5221 by the extraction of bacterial DNA with the All-4-One DNA extraction kit with the added step of lysozyme digestion (BIO BASIC Int., Markham, Canada) followed by real-time PCR with species-specific primers.

Metabolic marker determination: Weight, glucose, trehalose, triglycerides

Body weight was assessed by weighing ten flies in replicates of five. Glucose and trehalose (the glucose storage molecule in Drosophila) measurements were taken from both hemolymph and whole-body homogenates of Drosophila. For the whole-body homogenates, the total protein content was determined using a Bradford Assay and resultant quantification of metabolic markers was standardized against the total protein content in order to account for variations in fly mass. Following, the homogenate was heat-treated for 20 min at 70°C to remove any complexes. Glucose levels were measured in 2 μl of hemolymph or 5 μl of whole-body homogenate using the Glucose (HK) Assay kit (Sigma, Oakvilla, ON, Canada) according to the manufacturer's instructions. Whole-body trehalose was determine by digesting 2 μl of hemolymph or 5 μl of whole-body homogenate with 0.4 μl or 1 μl of trehalase (Sigma, Oakville, ON, Canada), respectively at 37°C for 4 h. After incubation, total glucose levels were measured with the Glucose (HK) Assay kit and the total amount of digested trehalose was determined by subtracting the values for total glucose. Whole-body triglycerides were determined in 10 μl of homogenate using the Triglycerides Liquicolor Test Mono (Stanbio, TX, USA) according to the manufacteur's instructions.

RNA isolation and real-time PCR

RNA was extracted from whole flies using Trizol (ThermoFisher, MA, USA) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA measured with the ND-2000 Nanodrop (FisherScientific, Ottawa, ON, Canada) using the High-Capacity cDNA Synthesis Kit (ThermoFisher, MA, USA) according to the manufacturer's instructions. Real-time PCR was conducted on the Eco Real-time PCR System (Illumina, CA, USA) using SybrGreen detection method (Diamed, Mississauga, ON, Canada). Primer pairs and annealing temperatures are listed in Table 1.

Statistics

All experiments were conducted in 3 independent trials with $n=5$ replicates in each experiment. Variations in body weight and motility over the dose-curves of probiotic were assessed via one-way ANOVA analyses with Tukey post-hoc tests. Significance of metabolic markers and genetic expression was assessed with two-way ANOVA with

Gene Name	Sequence	Annealing Temperature	References
Dilp 2	*forward: 3'* – AGCAAGCCTTTGTCCTTCATCTC – 5'	50°C	[21]
	reverse: 3' – ACACCATACTCAGCACCTCGTTG – 5'		
Dilp 3	*forward: 3'* – TGTGTGTATGGCTTCAACGCAATG – 5'	50°C	[21]
	reverse: 3' – CACTCAACAGTCTTTCCAGCAGGG – 5'		
Dilp 5	*forward: 3'* – GAGGCACCTTGGGCCTATTC – 5'	57°C	[21]
	reverse: 3' – CATGTGGTGAGATTCGGAGC – 5'		
ACC	*forward: 3'* – TTAGTCAGCTGCAGGCAAAGG – 5'	54°C	[22]
	reverse: 3' – CGGAAGCTAACGCCACACA – 5'		
FAS	*forward: 3'* – CAACAAGCCGAACCCAGATCTT – 5'	50°C	[22]
	reverse: 3' – CAAAGGAGTTCAGGCCGATGAT – 5'		
PEPCK	*forward: 3'* – CGCCCAGCGACATGGATGCT – 5'	60°C	[22]
	reverse: 3' – GTACATGGTGCGACCCTTCA – 5'		
dTOR	*forward: 3'* – GGCCGTCCAGGTTCAAAAAC - 5'	59°C	*This study*
	reverse: 3' – AATCCGGCGATAGTTCCGTC – 5'		
dAkt	*forward: 3'* – GAGTCGTGTGCTCAAGTCCA – 5'	59°C	*This study*
	reverse: 3' – TGCATCACAAAACACAGGCG – 5'		
dFOXO	*forward: 3'* – TCGCCGAACTCAGTAACCAC – 5'	59°C	*This study*
	reverse: 3' – TCCTATCAAAGTAGAGGCGCA – 5'		
Rp49	*forward: 3'* – AGATCGTGAAGAAGCGCACCAAG – 5 '	52°C	[23]
	reverse: 3' – CACCAGGAACTTCTTGAATCCGG – 5'		
Lf5221	*forward:* 3' – AGTCACGGCTAACTACGTGC– 5'	59°C	*This study*
	reverse: 3' – TCTACGCATTCCACCGCTAC– 5'		

Table 1: Primer pairs and annealing temperatures.

Tukey post-hoc analyses. Significance was determined if $p<0.05$ and evaluation at $p<0.01$.

Results

Orally administered Lactobacillus fermentum self-replicates in the Drosophila colon

L. fermentum NCIMB 5221 (Lf5221) is not an endogenous bacterial species in Drosophila although other Lactobacillus species including *L. plantarum* and *L. brevis* are. To determine if Lf5221 can self-replicate in the Drosophila colon, flies were inoculated with various concentrations of Lf5221 for 24 h and then transferred daily to new sterile media. Using real-time PCR, a baseline of Lf5221 was observed in the control group, likely indicating some non-specificity in the primer pairs for the commensal Lactobacillus species. Nevertheless, there was a dose-dependent increase in Lf5221 present in the colon dependent on the orally inoculated dose and these concentrations either remained the same (5.0×10^8 CFU/ml) or increased slightly over time for the 2.5×10^9 CFU/ml ($F(1,9)=5.34$, $p<0.05$) and the 7.5×10^9 CFU/ml group ($F(1,9)=6.43$, $p<0.05$), (Figure 1). Since there was an apparent increase in bacterial species over time without exogenous supply, it can be concluded that Lf5221 can self-replicate within the conditions of the Drosophila colon.

L. fermentum rescues physiological markers of diabetes in Drosophila fed high-sugar diet

High body weight is indicative of dysregulated energy metabolism and a state often materializing with diabetes. Flies fed on a high-sugar diet (HSD; 1.0 M sucrose) experienced a 26% increase in body weight ($p<0.01$) compared to flies fed a normal diet (0.15 M sucrose). However, co-feeding flies with Lf5221 rescued the weight gain in a dose-dependent manner such that at the highest tested concentration (7.5×10^9 CFU/ml) there was no difference in the average weight of flies fed a HSD compated to controls ($p>0.05$; Figure 2a). Flies fed on a HSD supplemented with heat-inactivated Lf5221 did not have a reduction in weight gain concluding that the metabolic activity of *L. fermentum* is necessary for the weight preservation effects.

Circulating levels of glucose (Figure 2b) and trehalose (Figure 2c) were similarly elevated in flies fed a HSD (>50% and 200%, respectively; $p<0.01$). However, neither living nor heat-inactivated Lf5221 significantly rescued circulating glucose or trehalose levels (Figures 2b and 2c; $p>0.05$).

Whole body glucose and trehalose are indicative of the physiological storage of glucose. Flies fed on a HSD experienced elevated whole-body glucose (>45%; Figure 2d, $p<0.01$) and whole-body trehalose levels (>33%; Figure 2e, $p<0.01$). Co-treatment with 5.0×10^8 CFU/ml Lf5221 reduced the amount of whole-body glucose by 25% from HSD-treated flies (Figure 2d; $p<0.01$) whereas at 7.5×10^9 CFU/ml, trending towards the same level as controls (Figure 2d, $p=0.06$). The main storage carbohydrate in flies is trehalose (comparable to glycogen in humans) and the elevated trehalose levels in response to the HSD was dose-dependently reduced by Lf5221 to a level comparable to controls at 5.0×10^8 CFU/ml (Figure 2e; $p<0.05$), while heat-inactivated Lf5221 did not affect whole-body trehalose levels.

Finally, whole-body triglycerides are also typically elevated in animals fed a HSD due to the broad dysregulation of energy metabolism. Likewise, flies fed on a HSD experienced almost a 50% increase in body weight (Figure 2f; $p<0.01$) with respect to controls. In a dose-dependent manner, Lf5221 reduced the total triglyceride concentrations, where at 7.5×10^9 CFU/ml reduced triglyceride concentrations to the level of control (Figure 2f; $p>0.05$).

Ferulic acid produced by *L. fermentum* NCIMB 5221 rescues diabetic markers in Drosophila fed a high sugar diet

Flies were reared on media containing various concentrations of FA and remained on the normal media containing FA for 7 days before being transferred to the HSD, also supplemented with FA. After three weeks, the same parameters as outlined for Lf5221 were measured. Flies grown on a HSD showed a 32% increase in body weight, which was reduced when flies were reared on media containing 0.1 mM FA (Figure 3a; $p<0.01$). In contrast to Lf5221, the elevation in circulating
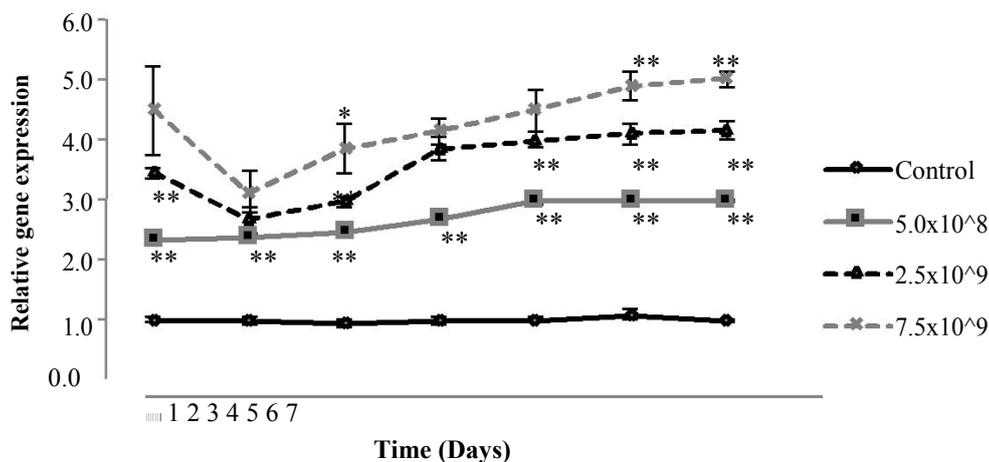

Figure 1: *L. fermentum* NCIMB 5221 persistence in the Drosophila colon after oral infection.

Flies were orally infected with four concentrations of *L. fermentum* NCIMB 5221 (CFU/ml) (Control – solid black lines with diamonds), 5.0×10^8 CFU/ml (grey lines with squares), 2.5×10^9 CFU/ml (black dotted lines with diamonds) and 7.5×10^9 CFU/ml (dotted grey lines with crosses) for 24 h. Flies were transferred to sterile bottles daily and sampled. Total *L. fermentum* NCIMB 5221 counts were assessed with real-time PCR of species-specific primers. Each sample represents $n=5$ replicates and it represented as the average gene expression +/- standard error. Error points * $p<0.05$ and ** $p<0.01$ represent the difference between the indicated dose point, and the point of the one-step lower concentration.

glucose levels (almost doubled in HSD-treated flies) was significantly reduced by 50% at 0.5 mM FA (Figure 3b; $p<0.05$). The effect of FA was even more pronounced for circulating trehalose where FA completely rescued the elevation in trehalose at 0.1 mM (Figure 3c; $p<0.01$). FA also had similar effects as Lf5221 for the whole-body glucose and trehalose levels. At 0.1 mM and 0.5 mM, FA rescued the 45% HSD-mediated elevation in total glucose (Figure 3d; $p<0.01$) and trehalose (Figure 3e; $p<0.05$) levels, respectively. Finally, it was further demonstrated that FA rescued the elevated total triglycerides levels in HSD-treated flies at 0.5 mM (Figure 3f; $p<0.05$). Interestingly, it appears that 0.5 mM is an optimal dose of FA and that above this amount (1.0 mM) there is a less beneficial effect on the diabetic parameters of HSD-treated flies.

Dilp expression is altered by ferulic acid producing *L. fermentum* NCIMB 5221 in Drosophila fed a high-sugar diet

Drosophila insulin-like peptide (*dilp*) expression in Drosophila is modulated and regulated similarly to insulin signaling in mammals. Is it thus expected that *dilp* expression will increase in response to HSD equivalent to insulin-resistance in mammals. Indeed, an increase of approximately 2-fold for both *dilp 2* and *3* was noted in flies fed a HSD compared to controls (Figure 4; $p<0.05$), however there was an insignificant change in *dilp5* (Figures 4e and 4f; $p>0.05$). With Lf5221 co-treatment, there was a complete rescue in *dilp2* and *3* expression at the lowest Lf5221 dose 5.0×10^8 CFU/ml (Figures 4a and 4c; $p>0.05$) which was not apparent in heat-inactivated Lf5221-treated flies. FA
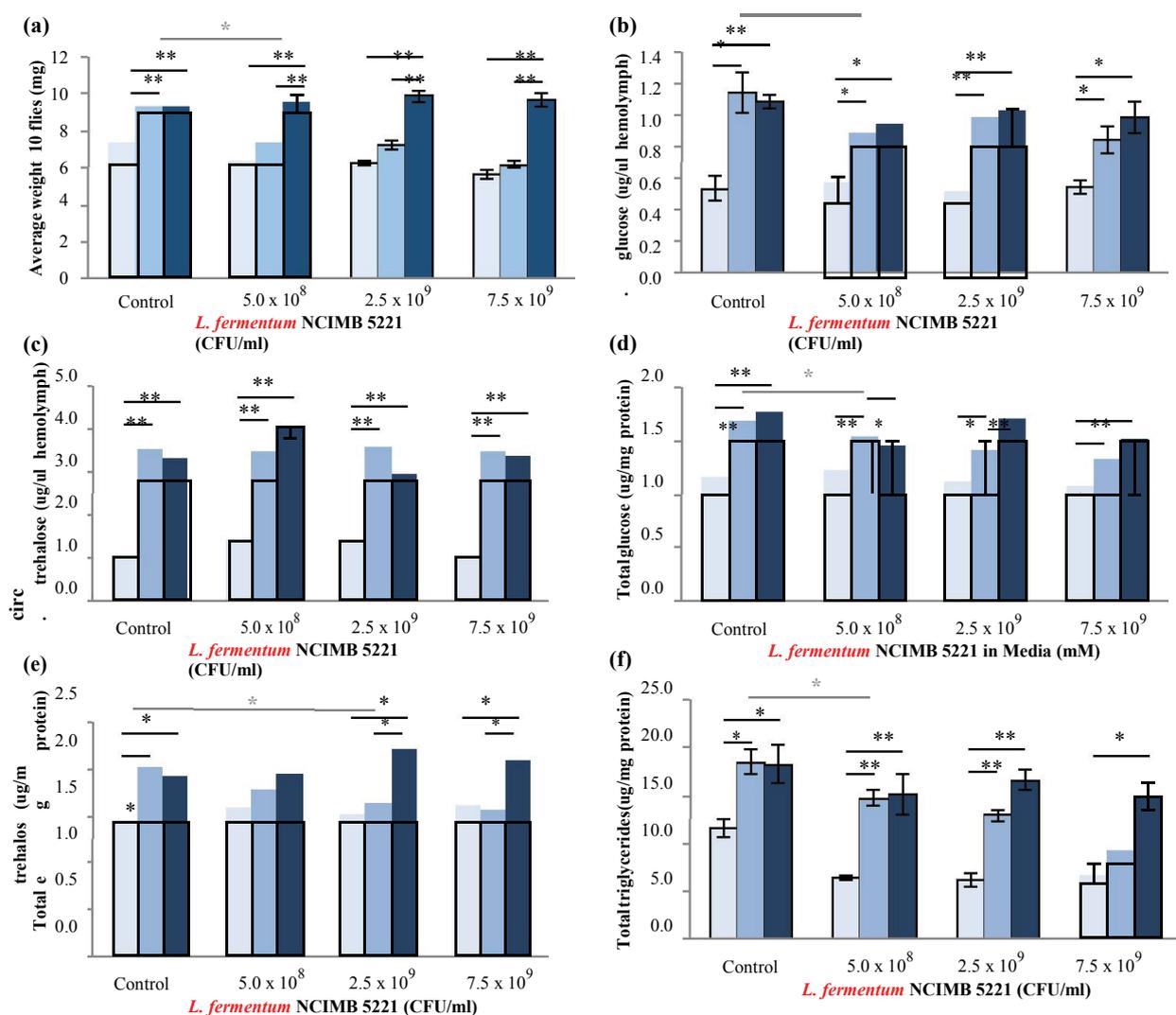

Figure 2: Metabolic markers of diet-induced diabetes are affected by oral treatment of the live probiotic *L. fermentum* NCIMB 5221. Flies were reared on media containing various concentrations of Lf5221. After eclosion, flies remained on the probiotic-inoculated media for 7 days and were then subsequently transferred to the high-sugar media (HSD) containing Lf5221 for 21 days to induce diabetes together with the indicated probiotic. All markers were measured in control (light-blue bars), HSD with live Lf5221 (medium-blue bars) and HSD with heat-inactivated Lf5221 (dark-blue bars) treated groups at doses of 0, 5.0×10^8, 2.5×10^9 and 7.5×10^9 CFU/ml. Various aspects of metabolic health were assessed including **(a)** average weight of 10 flies, **(b)** circulating levels of glucose normalized to l of hemolymph, **(c)** circulating levels of trehalose normalized to l of hemolymph, **(d)** whole-body glucose normalized to mg of protein, **(e)** whole body trehalose normalized to mg of protein and **(f)** whole body triglycerides also normalized to mg of protein. All values represent $n=5$ independent experiments +/- the standard error. Significance is indicated as * $p<0.05$ or ** $p<0.01$ where black lines indicate difference between treatments groups and grey lines indicate the first significance difference between dose groups for the HSD supplemented with live Lf5221.
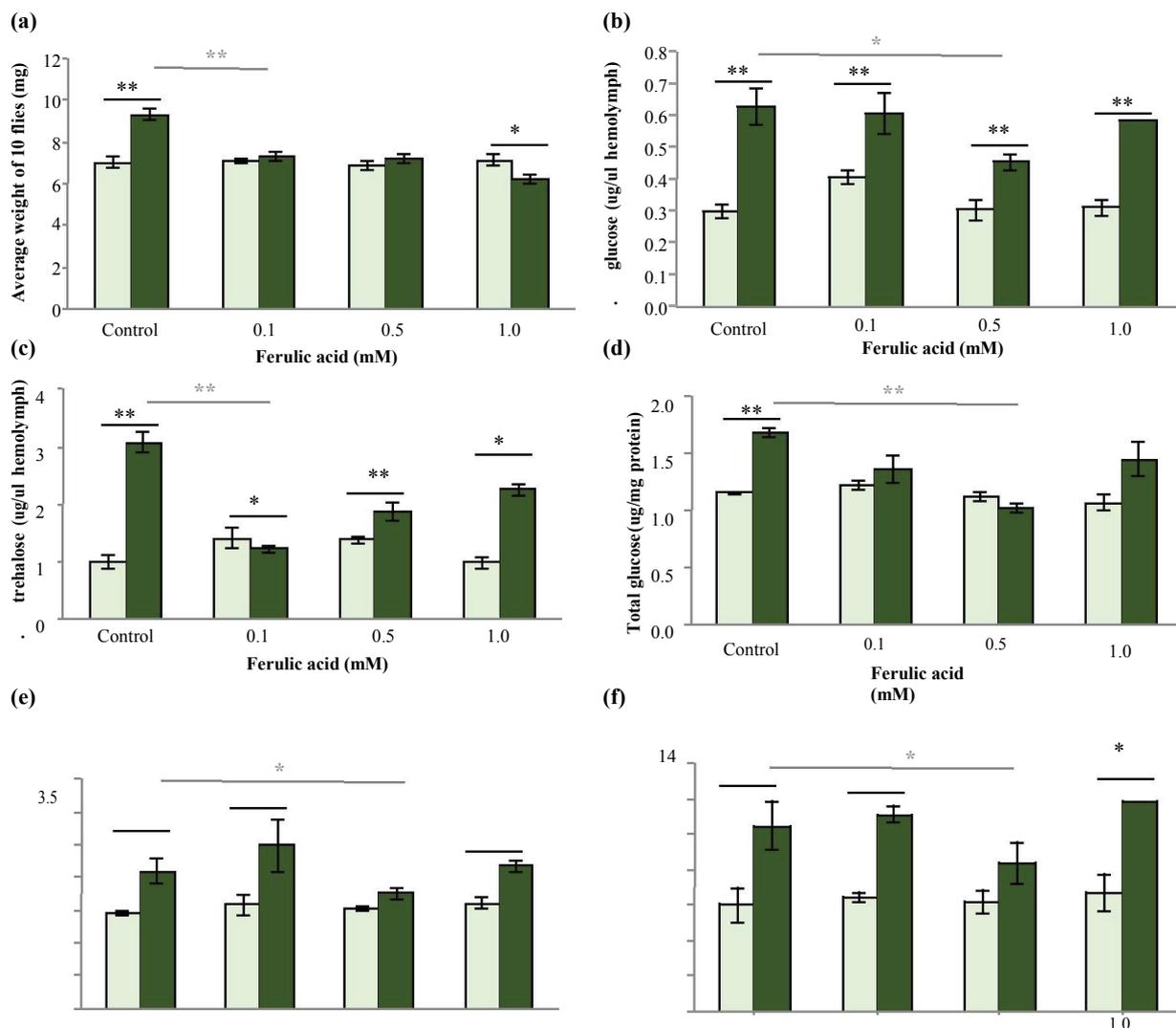
Figure 3: Implications of ferulic acid in energy metabolism of high-sugar fed Drosophila.
Drosophilas were reared on media supplemented with FA. After eclosion, flies remained on the FA-inoculated media for 7 days before being transferred to normal media supplemented with FA (light green bars) or a high sugar diet (HSD) media supplemented with FA (dark green bars). Various aspects of metabolic health were assessed including **(a)** average weight of 10 flies, **(b)** circulating levels of glucose normalized to l of hemolymph, **(c)** circulating levels of trehalose normalized to l of hemolymph, **(d)** whole-body glucose normalized to mg of protein, **(e)** whole body trehalose normalized to mg of protein and **(f)** whole body triglycerides also normalized to mg of protein. All values represent $n=5$ independent experiments +/- the standard error. Significance is indicated as * $p<0.05$ or ** $p<0.01$ where black lines indicate difference between treatments groups and grey lines indicate the first significance difference between dose groups for the HSD supplemented with FA.

similarly instigated a drastic rescue of *dilp* expression in HSD-treated flies at the lowest concentration, 0.1 mM (Figures 4b and 4d; $p<0.05$). Interestingly, FA also reduced *dilp5* expression mostly efficiently at 0.1 and 0.5 mM (Figure 4f; $p<0.01$) in contrast to the Lf5221 group which did not have a significant effect on *dilp5* expression.

FA-producing *L. fermentum* NCIMB 5221 reduced obesity markers in Drosophila fed a high-fat diet

Drosophila reared on a media supplemented with various concentrations of Lf5221 were allowed to age for 7 days before being transferred to a high-fat diet (HFD) media containing 20% coconut oil along with the identified concentrations of Lf5221. After three weeks, the mass of flies on the HFD was significantly elevated by 32% as compared to controls (Figure 5a; $p<0.01$). Flies co-treated with Lf5221, but not heat-inactivated Lf5221, demonstrated a dose-dependent decrease

in body mass where at a dosage of 2.5×10^9 CFU/ml had a reduction of 35% (Figure 5a; $p<0.05$). Total glucose and trehalose levels where significantly increased by 27% (Figure 5b; $p<0.05$) and 48% (Figure 5c; $p<0.01$), respectively. At 5.0×10^8 CFU/ml, Lf5221 rescued total glucose and trehalose levels to that of controls ($p>0.05$) where in both measures, the heat-inactivated Lf5221 has no effect. Whole-body triglycerides were significantly elevated by 50% in HFD treated flies as expected (Figure 5d; $p<0.01$), and dose-dependently reduced in Lf5221-treated flies reaching the level of controls at 2.5×10^9 CFU/ml (Figure 5d; $p<0.01$).

Ferulic acid from *L. fermentum* NCIMB 5221 reduced markers of obesity in high-fat diet fed Drosophila

Flies reared on media containing various concentrations of FA were allowed to age 7 days before be transferred to the previously described HFD. As expected, there was a 62% increase in body weight (Figure
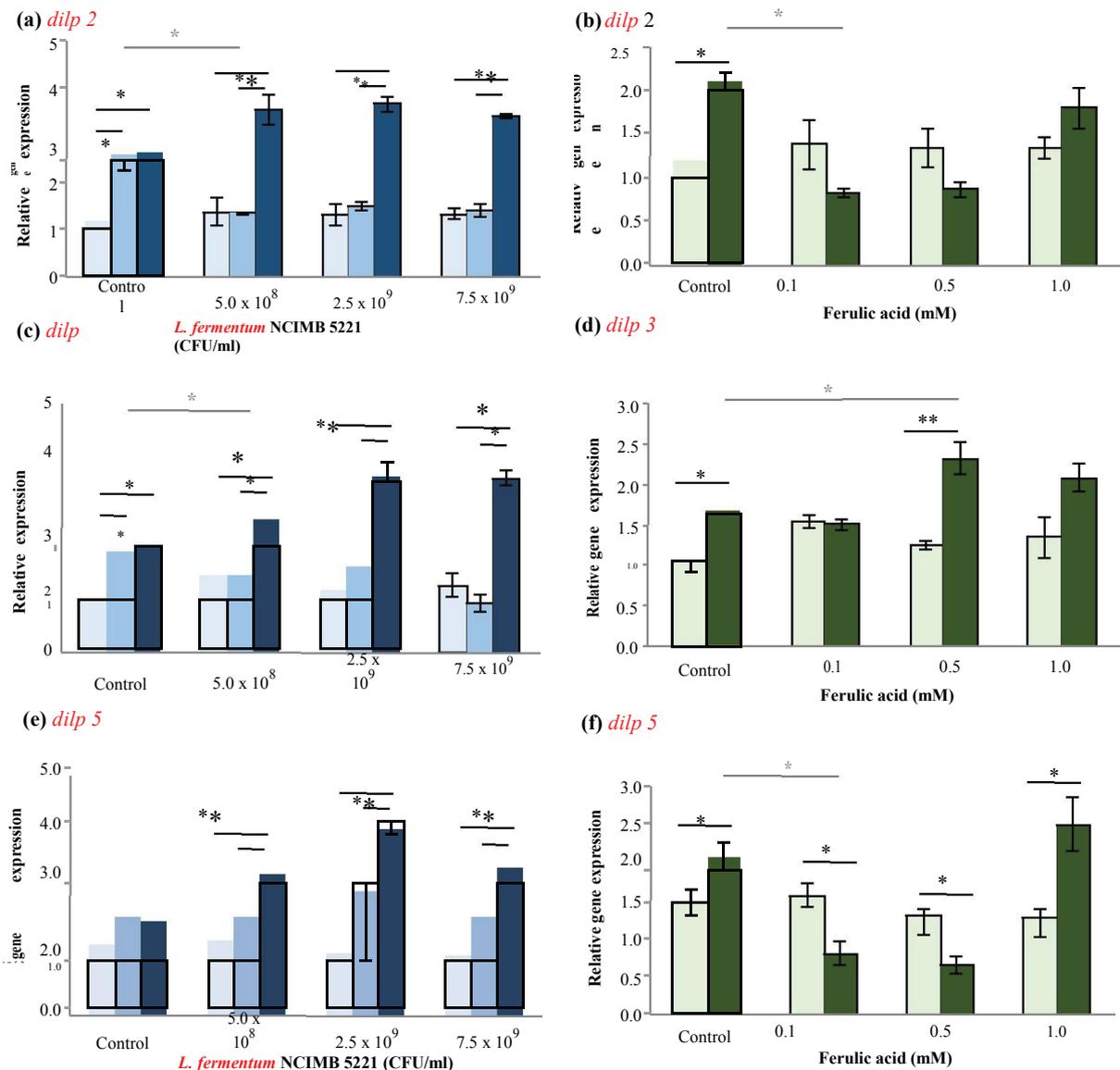
Figure 4: Expression of Drosophila insulin-like peptide (*dilp*) genes is influenced by *L. fermentum* NCIMB 5221 and ferulic acid in flies fed a high-sugar diet. Drosophilas were reared on media containing either Lf5221 or FA. After eclosion and 7 days of aging on the treated media, flies were transferred to treated high-sugar diet (HSD) media. Media was treated with either Lf5221 **(a,c,e)** with control media with live Lf5221 (light-blue bars), HSD with live Lf5221 (medium-blue bars) and HSD with heat-inactivated Lf5221 (dark-blue bars) or FA **(b,d,f)** with control media supplemented with FA (light green bars) or a HSD supplemented with FA (dark green bars). Expression of **(a,b)** *dilp 2* **(c,d)** *dilp 3* or **(e,f)** *dilp 5* was assessed with real-time pcr. Each point represents *n*=5 independent replications +/- standard error. Significance is indicated as * *p*<0.05 or ** *p*<0.01 where black lines indicate difference between treatments groups and grey lines indicate the first significance difference between dose groups for the HSD supplemented with Lf5221 or FA.

6a; *p*<0.01). At a concentration of 0.5 mM, FA maximally reduced the weight gain by 25% (*p*<0.01). There were also significant increases in both total glucose (Figure 6b) and trehalose (Figure 6c), which were significantly reduced by FA at 0.1 mM (*p*<0.01) and 0.5 mM (*p*<0.05), respectively. Finally, there was a significant 60% increase in whole-body triglycerides that was significantly reduced by 32% with supplementation of 0.5 mM FA (Figure 6d; *p*<0.01).

Fundamental gene expression of genes regulating fatty acid metabolism is altered by ferulic acid produced by *L. fermentum* NCIMB 5221

Acetyl CoA carboxylase (ACC), fatty acid synthase (FAS) and phosphoenolpyruvate carboxykinase (PEPCK) are three enzymes critical for the production of fatty acids (ACC and FAS) and glyceroneogenesis (PEPCK). All three multi-unit enzymes should be significantly elevated in HFD flies reflecting the increase in fatty acid production. ACC was significantly elevated in HFD flies, and reduced with treatment of 5.0×10^8 CFU/ml Lf5221 (Figure 7a; *p*<0.01) but not FA (Figure 7b; *p*>0.05). FAS was similarly elevated, yet significantly reduced by both Lf5221 at 5.0×10^8 CFU/ml (Figure 7c; *p*<0.01) and FA at 0.5 mM (Figure 7d; *p*<0.01). Finally, PEPCK was also elevated in HFD-fed Drosophila and significantly reduced by both Lf5221 at 2.5×10^9 CFU/ml (Figure 7e; *p*<0.01) and by FA at 0.5 mM (Figure 7f; *p*<0.01).
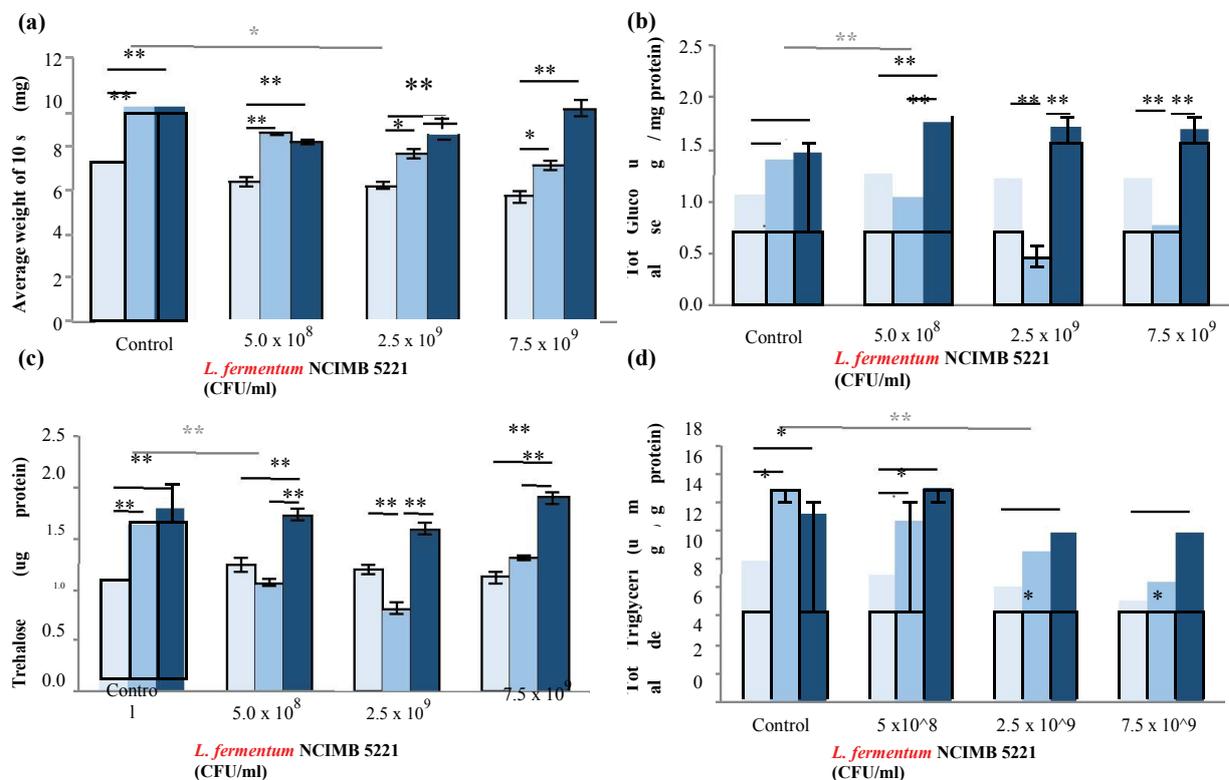
Figure 5: Implications of *L. fermentum* NCIMB 5221 in energy metabolism of high-fat fed Drosophila.
Drosophila*s* were reared on media containing various concentrations of Lf5221. After eclosion, flies remained on the Lf5221-inoculated media for 7 days and were then subsequently transferred to the high-fat diet (HFD) media containing Lf5221 for 21 days to induce obesity. All markers were measured in Lf5221 inoculated control (light-blue bars), HFD with live Lf5221 (medium-blue bars) and HFD with heat-inactivated Lf5221 (dark-blue bars)
treated groups at doses of 0, 5.0×10^8, 2.5×10^9 and 7.5×10^9 CFU/ml. Various aspects of metabolic health were assessed including **(a)** average weight of 10 flies, **(b)** whole-body glucose normalized to mg of protein, **(c)** whole body trehalose normalized to mg of protein and **(d)** whole body triglycerides also normalized to mg of protein. All values represent *n*=5 independent experiments +/- the standard error. Significance is indicated as * *p*<0.05 or ** *p*<0.01 where black lines indicate difference between treatments groups and grey lines indicate the first significance difference between dose groups for the HFD supplemented with Lf5221.

FA-producing *L. fermentum* NCIMB 5221 attenuates the fundamental mechanisms of IlS

To determine if the FA produced from Lf5221 affects the fundamental signaling mechanisms of insulin-like signaling (IlS), the genetic expression of dTOR, dAkt and dFOXO was analyzed by real-time PCR. As expected, HSD reduced the expression of the negative regulator of insulin, dFOXO while elevating dAkt and dTOR (Figure 8). In response to Lf5221, gene expression was rescued for dFOXO at 5.0×10^8 CFU/ml (Figure 8a; *p*<0.01), reduced for dAkt at 5.0×10^8 CFU/ml (Figure 8c, *p*<0.05) and rescued for dTOR at 5.0×10^8 and 7.5×10^9 CFU/ml (Figure 8e; *p*>0.05). Similarly for FA, dFOXO expression was rescued at 0.5 mM FA (Figure 8b; *p*>0.05), dAkt was rescued at 1.0 mM (Figure 8d; *p*>0.05) and dTOR was rescued at 0.5 mM (Figure 8f; *p*>0.05). This indicates that the rescued effects of HSD on Drosophila from orally-treated Lf5221 and FA is due to its modulatory action on fundamental IlS pathways.

Discussion

Metabolic syndrome is a collection of diverse metabolic phenotypes including insulin resistance, an increase in abdominal body fat, increased blood pressure, and abnormal cholesterol and triglyceride levels [2]. Current therapies include statins to control cholesterol, ACE inhibitors to lower blood pressure and various anti-diabetic medications. These

are all limited approaches to treating this multi-faceted disease as they only target one of the symptoms of metabolic syndrome, do not address the underlying cause and elicit several unwanted side effects. In the current study, the application of a single probiotic bacterium, *L. fermentum* NCIMB 5221 (Lf5221) towards the reduction of several metabolic syndrome markers was assessed. It was found that through the activity of Lf5221's metabolic product, ferulic acid (FA), markers of both diabetes and obesity were rescued to levels of control both on physiological and molecular levels possibly through the regulation of downstream IlS signaling factors including dTOR.

Alone, Lf5221 fed to *Drosophila melanogaster* at the dosage range from 5.0×10^8 to 7.5×10^9 CFU/ml elicited a dose-dependent reduction in body weight and total glucose and trehalose levels in the diet-induced diabetic model and a reduction in the body weight, total glucose, trehalose and triglyceride levels in the diet-induced obesity model. In the diabetes model, the diabetic phenotypes were completely rescued at 2.5×10^9 CFU/ml, which is consistent with a physiological dose in both animals and humans [21-25]. Considering that the effect of Lf5221 on these physiological markers of diabetes and obesity is lost when flies are they are fed on heat-inactivated Lf5221, it can be deduced that a secreted metabolic product of Lf5221 is responsible for the effects and not a signaling cascade implemented through a surface ligand. One of the main secreted products from Lf5221 is FA, produced through its intrinsic ferulic acid esterase (FAE) activity.
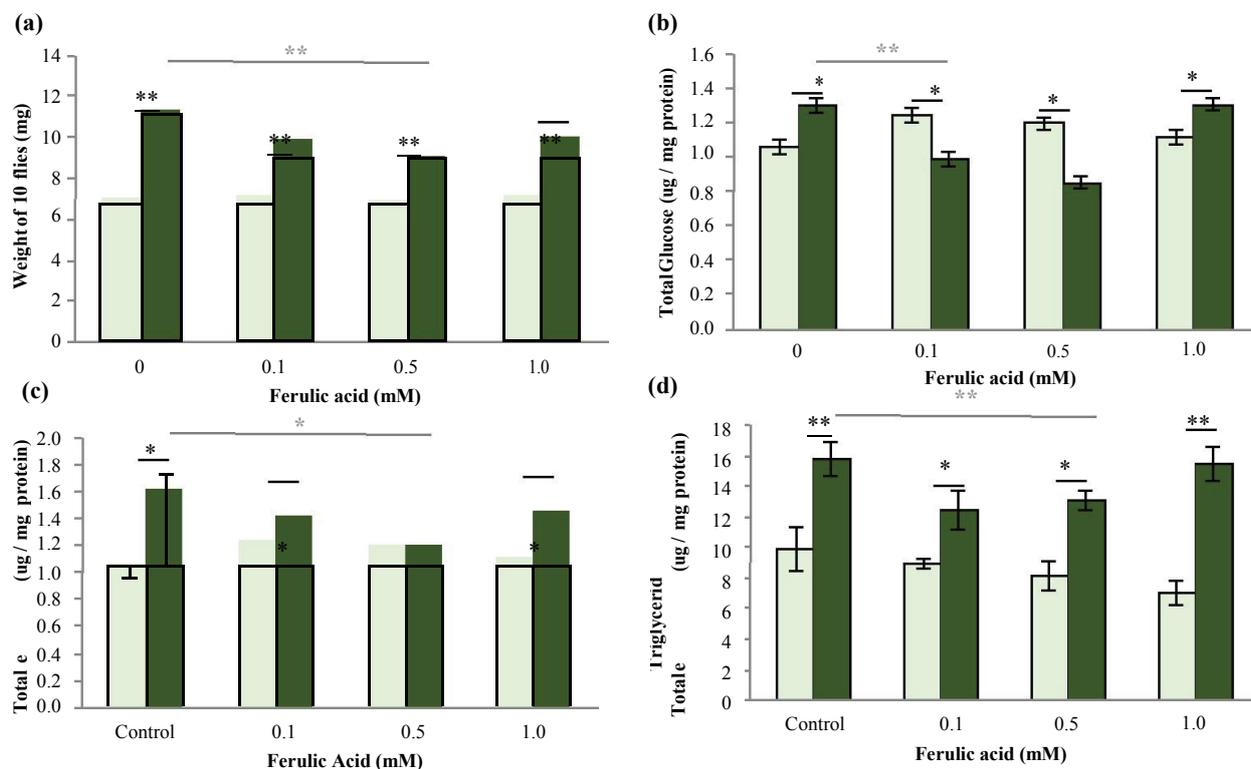
Figure 6: Implications of ferulic acid in energy metabolism of high-fat fed Drosophila.
Flies were reared on media containing various concentrations of ferulic acid. After eclosion, flies remained on the FA-inoculated media for 7 days and were then subsequently transferred to the high-fat diet (HFD) media containing FA for 21 days to induce obesity. All markers were measured in FA treated control (light-green bars), HFD supplemented with FA (dark-green bars). Various aspects of metabolic health were assessed including **(a)** average weight of 10 flies, **(b)** whole-body glucose normalized to mg of protein, **(c)** whole body trehalose normalized to mg of protein and **(d)** whole body triglycerides also normalized to mg of protein. All values represent n=5 independent experiments +/- the standard error. Significance is indicated as * p<0.05 or ** p<0.01 where black lines indicate difference between treatments groups and grey lines indicate the first significance difference between dose groups for the HFD supplemented with FA.

Previously, there have been several indications in human clinical trials that probiotics have a beneficial effect on various markers of metabolic syndrome including *B. lactis* [26], *L. plantarum* [27], *L. rhamnosus* [28], *L. gasseri* [29] and *L. reuteri* [25]. There are also conflicting studies showing that probiotics have a less beneficial effect on the aforementioned markers including *L. casei* Shirota [30]. Despite there being several studies indicating a beneficial effect of probiotics on one or more markers of metabolic syndrome, very few studies have addressed the underlying mechanisms of communication between the probiotic species and the host metabolic pathways.

Probiotics can alter the composition of the gut microflora in several ways including i) directly binding to the gut epithelial layer through surface ligands instigating downstream signaling cascades, ii) altering the broad composition of the microflora community in the GIT hence altering the efficiency of energy extraction from foods and mineral absorption and iii) producing bacterial metabolites that activate downstream signaling pathways that have broad implications on host physiology. In this study, the secreted metabolite of Lf5221, FA was specifically investigated in the context of metabolic syndrome to delineate a plausible mechanism of communication by Lf5221 to the metabolic health of the host.

FA is a well-known hydroxycinnamic phenolic phytochemical with anti-inflammatory and anti-oxidative activities [31]. It has also been previously shown that FA has several metabolic benefits. In a high-fat diet induced obesity model in mice, FA at doses of 25 and 50 mg/kg body

weight reduced elevated blood glucose and serum leptin levels, lowered the insulin resistance and increased the serum adiponectin levels, serum lipid levels and liver cholesterol and triglyceride accumulations. Further, this study found that FA reduced the expression of hepatic lipogenic genes such as sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) along with upregulating hepatic carnitine palmitoyltransferase 1a (CPT1a) and peroxisome proliferator activated receptor alpha (PPARα) [32]. FA at a dose of 2.6 mmol/kg diet was effective at reducing inflammatory, hyperlipidemia, hyperglycemia markers in a high-fructose, high-fat diet-induced model of metabolic syndrome [33]. A similar study found that FA minimized insulin resistance, vascular dysfunction and remodeling in a rat model of high-carbohydrate, high-fat diet-induced metabolic changes, which may be realized through suppression of oxidative stress, down-regulation of p47phox, increased nitric oxide bioavailability with up-regulation of endothelial nitric oxide synthase and suppression of tumor necrosis factor-α (TNF-α) [34]. It is clear from these studies that FA simultaneously targets several aspects of metabolic syndrome including hyperglycemic indices, hypercholesterolemia markers, inflammation, oxidation and others through impacting the fundamental signaling mechanisms underlying energy metabolism. This makes FA an ideal candidate to study the effects of metabolic syndrome.

In the present study, the metabolic action of ferulic acid was confirmed. FA at a dose of 0.5 mM rescued the circulating glucose and trehalose as well as whole-body glucose and trehalose in the HSD
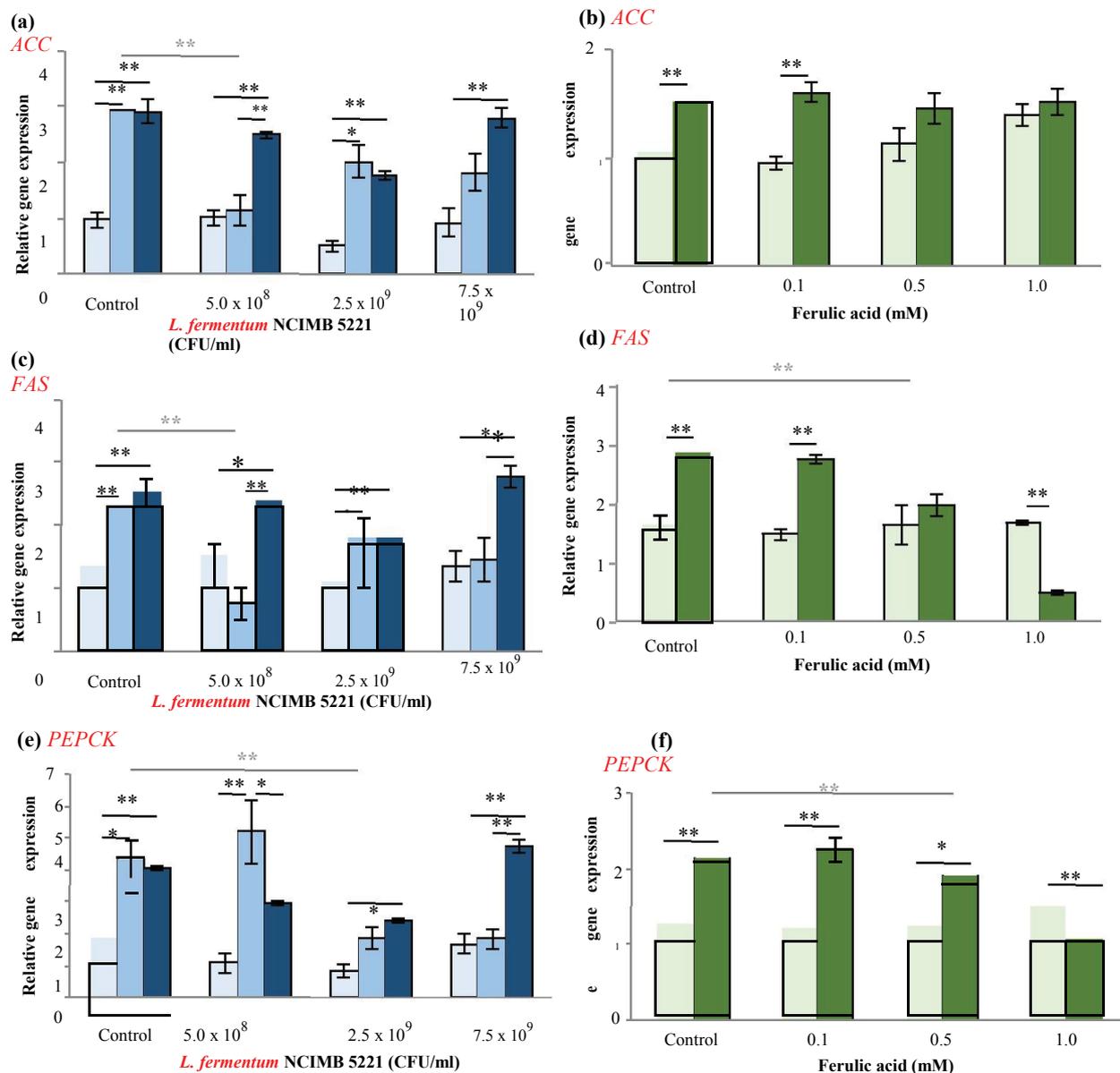
Figure 7: Expression of fatty acid metabolic genes in HFD fed flies exposed to *L. fermentum* NCIMB 5221 or Feurlic acid.
Drosophilas were reared on media containing either Lf5221 or FA. After eclosion and 7 days of aging on the treated media, flies were transferred to treated high-fat diet (HFD) media. Media was treated with either Lf5221 **(a,c,e)** with control media with live Lf5221 (light-blue bars), HFD with live Lf5221 (medium-blue bars) and HFD with heat-inactivated Lf5221 (dark-blue bars) or FA **(b,d,f)** with control media supplemented with FA (light green bars) or a HFD supplemented with FA (dark green bars). Expression of **(a,b)** *ACC* **(c,d)** *FAS* or **(e,f)** *PEPCK* was assessed with real-time pcr. Each point represents *n*=5 independent replications +/- standard error. Significance is indicated as * *p*<0.05 or ** *p*<0.01 where black lines indicate difference between treatments groups and grey lines indicate the first significance difference between dose groups for the HFD supplemented with FA.

fed flies. In the HFD flies, FA at a dose of 0.5 mM rescued the total glucose, trehalose and significantly reduced the total triglyceride levels as compared to untreated groups. Especially considering the effects on the circulating levels of glucose and trehalose, the isolated FA treatment seemed to be more effective than Lf5221. This could be due to the higher concentrations of ferulic acid reaching the various organ systems in the fly.

In mammals and flies alike, insulin resistance is determined through the overproduction of insulin and insulin-like growth factor signaling in response to circulating levels of glucose. In mammals, it is the expression of insulin-like receptor and glucagon-like peptide

that are altered when insulin resistance is developed. In flies, it is the production of the 8 *Drosophila insulin-like peptides* (*dilps*) and the Drosophila insulin receptor (DInR). It was found that signaling and activation of the DInR through tyrosine phosphorylation stimulates *dilp* 2 or *dilp* 5 in the insulin-producing cells in the Drosophila neural net [35]. Further, altered expression of the genes encoding *dilp* 2, 3, 5 and 6 results in modulated IlS and profound metabolic and longevity consequences [36]. In addition, nutrient status will activate *dilp* expression through the activation of *Unpaired* 2 (Upd2). The function of each of the *dilp* homologs has not been fully elucidated but it has been postulated that *dilp* 2 is involved in carbohydrate and trehalose
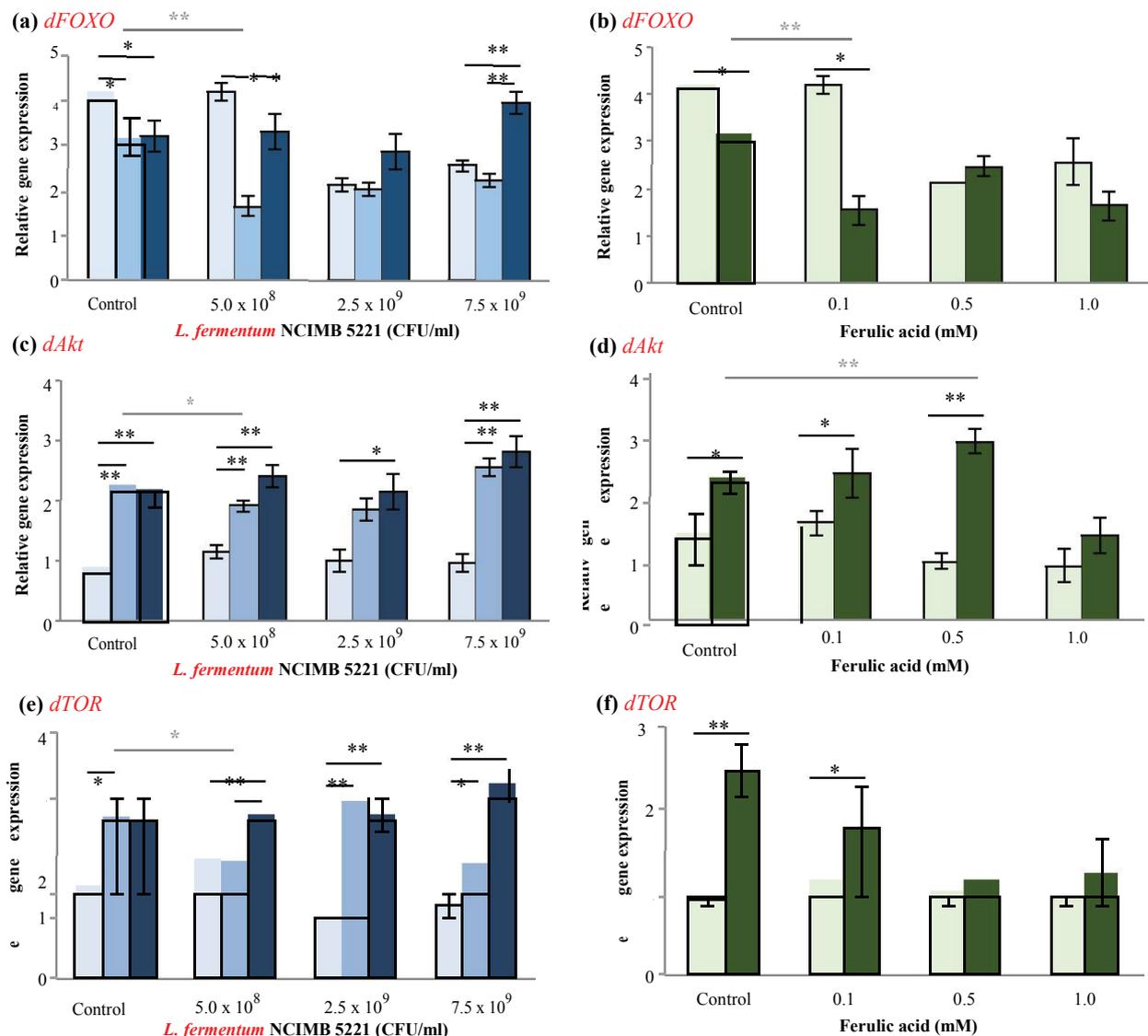
Figure 8: Expression of upstream regulators of insulin signaling in *Drosophila melanogaster* fed a HSD with *L. fermentum* NCIMB 5221 or ferulic acid. Drosophilas were reared on media containing either Lf5221 or FA. After eclosion and 7 days of aging on the treated media, flies were transferred to treated high-sugar diet (HSD) media. Media was treated with either Lf5221 **(a,c,e)** with control media with live Lf5221 (light-blue bars), HSD with live Lf5221 (medium-blue bars) and HSD with heat-inactivated Lf5221 (dark-blue bars) or FA **(b,d,f)** with control media supplemented with FA (light green bars) or a HSD supplemented with FA (dark green bars). Expression of **(a,b)** *dFOXO* **(c,d)** *dAkt* or **(e,f)** *dTOR* was assessed with real-time pcr. Each point represents *n*=5 independent replications +/- standard error. Significance is indicated as * *p*<0.05 or ** *p*<0.01 where black lines indicate difference between treatments groups and grey lines indicate the first significance difference between dose groups for the HSD supplemented with Lf5221 or FA.

metabolism, *dilp* 3 in lipid metabolism and *dilp* 5 in dietary restriction response and longevity [36].

As expected, there was a significant increase in *dilp* 2, 3 and 5 expression in flies fed a HSD. Lf5221, but not heat-inactivated Lf5221, efficiently reduced dilp 2 and 3 expression to the level of controls. Similar to the physiological results, FA was more efficient at reducing dilp 2, 3 and 5 expression levels at both 0.1 mM and 0.5 mM doses. This confirms that it is the metabolic activity of Lf5221 that is eliciting the gene-dependent regulation of dilps and not passive signaling, possibly through the action of FA. As previously outlined, it is not known at what level the gut microbiota, and its metabolites are influencing the regulation of insulin signaling. The downstream IIS signaling factors

are the same in Drosophila and mammals so the expression of three of the key mediators, dFOXO, dAkt and dTOR, was assessed.

In mammals and flies alike, Akt and TOR are induced downstream of insulin signaling, which subsequently suppresses FOXO [37]. Likewise, in flies fed on a HSD, there was a significant elevation in dAkt and dTOR, and a decrease in dFOXO. Notably, dFOXO and dTOR, but not dAkt expression was rescued at 2.5×10^9 CFU/ml Lf5221 treatment. Similarly, FA treatment at 0.5 mM rescued dTOR and dFOXO at 0.5 mM and dAkt at 1.0 mM. Notably, the downstream signaling factors dTOR and dFOXO were affected by Lf5221 treatment, but not dAkt indicating that the regulation of insulin signal in flies is controlled downstream of dAkt.

This result is significant as dTOR is the key energy-sensing molecule in the body acting as an integrator of intrinsic molecular and hormonal cues with environmental nutritional signals. It is well known that the mammalian mTORC is dysregulated in patients with diabetes, obesity and metabolic syndrome [38]. Through IlS pathways, TOR signaling (via the complex mTORC1 in mammals) is induced by the phosphorylation and inactivation of the upstream inhibitors of TOR, TSC1/2, by Akt. Hence, through Rheb signaling, TOR is disinhibited and allowed to bind to its effector co-factors. In contrast, mTORC1 is also inhibited by AMP-activated protein kinase (AMPK), an energy-sensing molecule that is activated in response to fluctuations in the AMP:ATP ratios. AMPK integrates several energy-regulating cues including glucose uptake, β-oxidation of fatty acids and the biogenesis of glucose transporters. Nutritionally, mTORC is directly regulated by high levels of free amino acids and growth factors including IGF-1/2 [39]. Interestingly, one study has indicated that the growth-promoting properties of a commensal gut microbiota *L. plantarum* in Drosophila, may act via a dTOR-dependent mechanism, though the method of communication between the gut microbiota and TOR signaling was not elucidated [40].

Interestingly, there have been some indications that ferulic acid can directly impact TOR signaling. For example, one study in HeLa cells showed that ferulic acid inactivated TOR signaling to a similar extent as the well-known inhibitor rapamycin [41]. In contrast, another study in a rodent ischemia model indicated that FA not only had a reduced infarct volume, but also reduced the injury-induced decreases in dTOR and its downstream effectors p70S6 kinase and S6 phosphorylation [42]. In addition, the anti-oxidant and anti-inflammatory actions of FA may also indirectly impact dTOR regulation, which is complex and involves several parallel factors. Nevertheless, the current study strongly suggests that the energy-regulating properties of Lf5221 occur through FA-mediated modulation of dTOR signaling.

One consideration for this study is the bioavailability of FA and the differences in its metabolism between flies and humans. FA is readily and rapidly metabolized by the gut microflora to generate simpler absorbable phenolic compounds [43] including dihydroferulic acid, vanillic acid, protocatechuic acid, p-coumaric acid and caffeic acid [44]. Indeed, it is the activity of these smaller polyphenols that are believed to elicit the anti-oxidant, anti-inflammatory and metabolic effects on the host [45]. However, it could alternatively be the bacterial metabolites secreted from the community of microbes in an environment enriched with the polyphenol elements that elicits the physiological effects. Regardless, the signaling pathways that are affected by the consumption of FA are dependent on the gut microbiota community composition, which varies from person to person. It is also dependent on the lability of the consumed polyphenol by transiting through the entire GIT subjected to the harsh acidity of the stomach and bile secretion in the small intestine. FA delivered orally is absorbed in the upper GI tract and rapidly excreted, providing only a short residence time [46,47] and bioavailability of FA is thus determined by its bioaccessibility [48] (e.g. bran bound FA is more bioavailable than free FA [49]. It is noted that free FA formulations are not available and attempts have been made to use FA rich food, modify FA and process food with very limited successes [47,50,51]. The use of a probiotic bacterium that produces FA continuously in the gut could potentially overcome this limitation while providing the synergistic benefits of a probiotic bacterium.

Altogether, consumption of an intrinsically FA-producing probiotic bacterium circumvents both of these issues by secreting FA locally in the GIT thus increasing the active concentration where it is most needed. Also, probiotic bacteria, especially was encapsulated

in microcapsules such as the alginate-poly-l-lysine-alginate capsules, has an increases bioavailability in the lower colonic regions where most of the beneficial bacteria that conduct the biotransformation of polyphenolic compounds are found [13].

Conclusion

Metabolically active probiotic bacteria are a viable option for the treatment of metabolic syndrome as they can simultaneously treat many if not all of the physiological manifestations of metabolic syndrome including hypercholesterolemia, hyperglycemia, insulin resistance, high blood pressure etc. In particular, the commensal FAE-active probiotic bacteria Lf5221 has been shown to be a potent producer of FA. The anti-inflammatory, anti-oxidative and metabolic activity of FA makes it a good therapeutic alternative to the treatment of metabolic syndrome. By supplementing ones diet with an environmentally resistant probiotic bacterium, the active component FA will be released in the lower colonic compartments where its activity is most beneficial circumventing its pending degradation in the harsh environments of the GIT. Further, the action of FA was shown to impact not only the physiological aspects of metabolic syndrome, but also the fundamental IlS cascades, possibly through the modulation of dTOR, an integrator of intrinsic energy regulators and environmental influences. This is highly significant as dTOR is intregral to several energy-regulating diseases including diabetes, obesity and even neurodegeneration. This makes Lf5221 a promising therapeutic agent for not only metabolic syndrome, but any energy-regulating disorder.

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