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Dairy Products Added in Media Affect the Development of *Drosophila melanogaster* (Diptera: Drosophilidae)

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Abstract

Due to the increased interest shown in kefir, the present study examined its effects on some developmental characteristics in *Drosophila*. To compare the results related to kefir, another fermented product, i.e., yogurt, and the raw material of kefir, i.e., milk, were included in the experiment. All three foods were fed to *Drosophila* by addition to the medium. The results showed that the three foods delayed development in generations F_1 and F_2. In both generations, the number of adults was lower than that of the control group, but the comparison of the foods among themselves showed no significant difference. Moreover, the negative effect continued in generation F_2. Phenotypically abnormal individuals were observed in the experiments, but there was no significant difference. Furthermore, the sex ratio changed in all the groups, including the control group. In the experimental group fed kefir, the sex ratio changed in favor of females, whereas in the other groups, it changed in favor of males. Whether the milk and milk products were fermented did not change the effect on *Drosophila*, but unexpectedly it delayed development, reduced the number of adults, and changed the sex ratio. It can be concluded that the three foods delayed development and altered the sex ratio in *Drosophila*.

Key words: kefir, yogurt, milk, *Drosophila melanogaster*, development

Kefir is attracting increasing interest due to its health-promoting effects, and a number of studies have confirmed these effects. Moreover, consumption has increased because it is a safe and cheap food that can be produced at home easily (Rosa et al. 2017). Kefir is a symbiotic association of lactic acid and acetic acid bacteria and lactose-fermenting yeasts obtained by fermenting milk (Dias et al. 2017). Studies in mice that showed antitumor activity and tumor development were considerably suppressed (Murofushi et al. 1986, de Moreno de LeBlanc et al. 2006, de Moreno de LeBlanc et al. 2007). Kefir was also reported to have a protective effect on tissue and serum functions (Yener et al. 2015), to aid in protein digestion, and to lower the glycemic index (Urdaneta 2007). The effect of kefir and β-glucan was investigated in production of broiler chickens, and the results suggested that their administration individually or in combination would improve growth performance and meat quality (Cho 2013). Despite the emergence of kefir among probiotic foods, there is little information in the literature about the safe level of consumption of kefir, the amount that should be consumed, the time it takes to show beneficial effects, etc. Based on increasing kefir consumption due to the globalization of daily food habits, research is urgently needed on these subjects (Rosa et al. 2014). The majority of the research was conducted in humans and on mice, with apparently no studies being done on insects.

Yogurt is a milk derivative–like kefir that is produced by fermentation of milk and is widely consumed globally. For this reason, it was used in the present study to compare it with kefir and as a second fermented product. Traditionally, yogurt is produced using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* bacterial cultures and it is rich in protein, calcium, vitamin D, riboflavin, and vitamins B6 and B12 (Holden et al. 2008). Lactic acid bacteria and yeasts are the main group of microorganisms associated with traditional fermented foods. Probiotics are microorganisms that benefit host health when consumed in sufficient quantities and they have significant effects on the intestinal flora. Recognition of the importance of intestinal flora continues to rise (Kabak and Dobson 2011). Animal studies have confirmed that probiotic bacterial yogurt and fermented milk inhibit tumor formation and proliferation. Reddy et al. (1983) reported that yogurt-fed mice had a 28–35% reduction in tumor cells compared with control groups fed milk. Probiotic bacteria have been shown to protect against cancer in the gastrointestinal tract (Goldin et al. 1984, Lidbeck et al. 1991). Furthermore, the immunity-stimulating effect of lactic acid bacteria was investigated in silkworms, and it was found that mortality was lower in silkworms infected with *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus mundtii* that were fed yogurt (Nishida et al. 2016).

Kefir and yogurt are obtained by the fermentation of milk and so milk was added to the test set in order to be able to compare the effects of milk and fermented products on *Drosophila melanogaster* (Diptera: Drosophilidae). Milk is a very rich source of biologically

active molecules, known to support development, improve immunity, reduce osteoporosis, and have positive effects on infectious and inflammatory diseases (Heaney 2009, Hill and Newburg 2015). It has been reported that regular consumption of lutein-rich fermented milk enhances the DNA repair capacity of lymphocytes (Herrero-Barbudo et al. 2013). Research on nutritional conditions affecting egg production in *Musca domestica* found that when milk was added to the adult diet as a protein source, the number of eggs per day and weight of the eggs increased (Pastor et al. 2011).

In the present study, the effects of kefir and yogurt on some developmental characteristics of *D. melanogaster* were investigated due to the recent increase in interest in fermented products and the lack of studies on their in vivo effects in insects, despite this increased interest. Model systems are needed to learn the principles governing the dynamics and functions of microbial communities in order to establish the details of the relationship between microbiology and disease. *D. melanogaster*, thanks to its short breeding period and cultivable microbiota, provides an ideal model for examining the dynamics of the microbiota throughout its host's life span (Blum 2013). Besides *D. melanogaster* being homologous to 60% of human disease genes (Schneider 2000), it is also a good model organism to investigate organism biology (Ormerod et al. 2017). *Drosophila* has almost all of the basic metabolic activation enzymes that mammals have, and thus, it is an organism commonly used in genetic and toxicology studies (Pandey and Nichols 2011). It would be beneficial to investigate the effects of microorganisms in food in in vivo conditions and in different experimental animals. Some bacterial strains could be tested for beneficial or detrimental effects on the fly's physiology or developmental responses. For this purpose, the effects of kefir, yogurt, and milk on the development of *Drosophila* were investigated in the present study.

Materials and Methods

The highly inbred Oregon-R strain of *D. melanogaster* was used. The *Drosophila* cultures were maintained in the dark and 40% relative humidity in a refrigerated incubator set to 25 ± 1°C. Instant *Drosophila* medium (Carolina Formula 4–24 Drosophila Medium) was used. This medium, which is sold with all ingredients ready to use, is prepared in 2 to 3 min by adding 15 ml of water and 15–20 grains of brewer's yeast to 5 g of instant dry medium, without the need for cooking or sterilization. It is used in *Drosophila* experiments worldwide to save time and ensure standard conditions, and its main ingredients are corn flour, agar, and brewer's yeast. In the experimental groups, kefir, yogurt, and milk were added instead of water to the medium. Water was added to the control group. The amount of water added to the control group (15 ml) was equal to the amounts of kefir, yogurt, and milk added to the experimental groups. To determine the lethal concentrations of milk and fermented products at the beginning of the study, nutrients were added to the medium in amounts ranging from 1 to 15 ml, and 100 adults (50 females + 50 males) were placed in each bottle, but no adult deaths were observed. This is the reason why the amounts of kefir, yogurt, and milk applied to the experimental groups were the same as the amount of water added to the control group. The milk used was natural cow's milk produced locally in Anatolia. The kefir was produced by traditional fermentation from the same milk and filtered. The yogurt was also fermented traditionally using the same milk. Natural milk was used and the kefir and yogurt were fermented by traditional methods in the home in order to determine the effects of completely natural products. The aim here was to observe the effect of microorganisms found in natural products only, eliminating the

effects of shelf-life-enhancing and preserving additives during the commercial production of yogurt, kefir, and milk.

For the application of kefir, yogurt, and milk, 10 males and 10 females were placed as parents in the experimental and control group bottles and individuals that developed from eggs formed the F_1 generation. It was noted that parental females and males selected for cross-breeding should be 3–4 d old. After crossing-breeding, the bottles were checked daily. When the development of pupae was observed, the parents were removed from the medium and only F_1-generation individuals were left. The morphology of each individual was examined and the observed phenotypic abnormalities and sex were recorded every day for 8 d, starting from the day when the first adult emerged, by separating the females and males under a dissecting microscope. Eight days from the day when the first adult was observed were chosen, because metamorphosis in *Drosophila* lasts 10 d. Continuing counts after 8 d may result in confusion between F_1 and F_2 results if it is thought that the egg of an F_1 adult laid on the first day will be an adult on the tenth day. The counts were terminated on the eighth day to prevent this confusion and separate the two generations precisely from each other. To observe the effect of milk and fermented milk products on the sex ratio, after cross-breeding, males and females obtained from both generations were recorded separately. The data were evaluated to determine whether there was a change in the sex ratio (Karataş and Bahçeci 2009b, Karataş et al. 2011a). An Olympus SZ61-dissecting microscope was used. However, since this microscope was not trinocular, photographs of abnormal phenotypes could not be taken. Twenty crossings were performed for both F_1 and F_2. The experimental bottles used were transparent glass bottles of 150-ml volume.

In the next stage of the study, the F_2 generation was observed to determine whether the possible effect of the substances applied continued in subsequent generations. For this purpose, 10 females and 10 males 3 to 4 d old randomly selected from the F_1 generation according to the substance applied (kefir, yogurt, milk, or water) were transferred to the substance-free medium. Thus, 10 females and 10 males randomly selected from the F_1 generation were transferred to the standard medium and cross-bred and the F_2 generation was obtained. The same process was carried out for the F_2 generation as for the F_1 generation during the observation phase (Karataş et al. 2011a). The *z*-test was used to compare the ratios of the statistical methods used in the evaluation of the data obtained (Ratkowsky 1990). The ratios were converted to *z*-points and the differences between the counts of two groups were tested as follows:

$$z = \frac{(p_1 - p_2)}{\sqrt{\frac{p_1 q_1}{n_1}}} + \left(\frac{p_2 q_2}{n_2}\right); q_2 = 1 - p_1.$$

Results

The results of adding kefir, yogurt, and milk to *D. melanogaster* medium are summarized in tables. Table 1 shows the number of adults in the F_1 generation. According to the data, milk, kefir, and yogurt, all inhibited development in *Drosophila*. The statistical difference between the three experimental groups and the control group was significant, but there was no difference in the comparison between the experimental groups. Therefore, whether milk and milk products were fermented did not change the effect on *Drosophila*, but unexpectedly it caused a decrease in the number of adults. An almost 50% decrease in the number of adults can be clearly seen in Table 1. In the F_2 generation as well, the number of adults in all the

Table 1. Effect of some foods on the number of adults in the F_1 generation

Groups	Total	Group comparisons	Z value
Milk	1317	G1-G2	0.735
(G1)		G1-G3	0.605
		G1-G4	27.115*
Yogurt	1351	G2-G3	−0.129
(G2)		G2-G4	26.358*
Kefir	1345	G3-G4	26.491*
(G3)			
Control	2721		
(G4)			

*$P < 0.01$.

Table 2. Effect of some foods on the number of adults in the F_2 generation

Groups	Total	Group comparisons	Z value
Milk	1626	G1-G4	15.387*
(G1)		G1-G2	7.070*
		G1-G3	0.765
Yogurt	1998	G2-G4	8.284*
(G2)		G2-G3	−6.303*
Kefir	1665	G3-G4	14.616*
(G3)			
Control	2464		
(G4)			

*$P < 0.01$.

experimental groups was significantly lower than that in the control group. This difference is particularly high in the experimental groups given milk and kefir (Table 2).

Tables 3 and 4 show the effect of kefir, yogurt, and water on the sex ratio of the F_1 and F_2 generations in *Drosophila*. In both generations, the sex ratio changed in all groups, including the control group, and the sex ratio of the experimental group fed kefir changed in favor of females, whereas in the other groups, it changed in favor of males.

Tables 5 and 6 show the numbers of individuals with abnormal phenotypes in the F_1 and F_2 generations and their comparisons. The resulting anomalies are various wing anomalies including bent wings, folded wings, shrunken wings, wings adhered to the body, and fringed wings. The rate of abnormalities in the experimental group fed yogurt in the F_1 generation was significantly greater compared with the other experimental groups but was still lower than that of the control group. In the F_2 generation, the percentage of abnormal individuals in any experimental group did not differ from that in the control group. Therefore, it can be concluded that milk, yogurt, and kefir did not cause abnormal individual development but unexpectedly did not prevent abnormal development, because the percentage of abnormal individuals in the experimental groups was not significantly different from that of the control group. Moreover, the tables show that the number of abnormal individuals is lower in both generations than in the control group, but the difference is not statistically significant.

Discussion

The results of the present study showed that kefir, yogurt, and milk reduced the number of adults in *Drosophila*, thereby impeding development. The observation of a decrease in the number of adults in *Drosophila* is usually a result of the application of toxic substances (Karataş and Bahçeci 2009a, Karataş et al. 2011a, Karataş et al. 2011b, Keser and Karataş 2012). Hence, it was unexpected that foods that are nutritious and beneficial for humans had such negative effects on *Drosophila* that they could be considered toxic. Kefir, yogurt, and milk negatively affected development in *Drosophila*, regardless of whether they were fermented or not. This negative effect was observed in the F_2 generation, which was not exposed to the F_1 generation or the food, and a decrease in the number of adults was observed. Since the number of abnormal individuals observed in the experimental groups was lower than that in the control group, it can be concluded that kefir, yogurt, and milk do not cause abnormal development of individuals but unexpectedly delay normal development. Findings related to the F_2 generation are important because it completes its development without being

subjected to the application of the food. Despite this, the number of adults in the F_2 generation was low. Recessive lethal mutations may be responsible for the reduction in the number of individuals in the F_2 generation when they are not exposed to the foods. At the beginning of the study, the toxicity of the lethal concentration cannot be detected during the course of the lethality test, and the reason may be the use of mature individuals in determining the lethal concentration. However, the F_1 generation is made up of eggs that have completed their development in the medium containing kefir, yogurt, and milk. Thus, the early developmental stages of an organism (egg and larval stage) are more susceptible to toxicity than the adult stage (El-Toukhy and Girgis 1993). This may be due to the large fall in the number of individuals in the F1 and F2 generations when toxic effects are not observed during the determination of lethal concentrations, because in the development of *Drosophila* larvae, yeast is the main protein source and it supports larval development as well as increasing egg production (Miller et al. 2011). Yeast growth may be adversely affected by the nutrients applied and the main protein source of the *Drosophila* larvae may have been reduced. The development of the larva may not have been completed because the addition of the three foods restricted the larvae's food. In addition, acetic acid bacteria are a group of bacteria that prefer fermented sugar and ethanol-rich environments (flower nectar, fruit, and vegetables) and can enter the *Drosophila* intestines from the environment (Blum et al. 2013, Shingleton et al. 2017). Acetic acid bacteria prevent growth of pathogenic bacteria in *Drosophila* and play a role in larval development rate, body size, intestinal stem cell activity, and energy metabolism (Blum et al. 2013). It has been reported that acetic acid bacteria are the major components of the microbiota of *Drosophila suzukii* and *D. melanogaster* and may play an important role in the physiology and behavior of the host (Vacchini et al. 2017). Kefir, yogurt, and milk added to the medium also resulted in the reduction of acetic acid bacteria taken from the environment into the intestines and the symbiotic relationship established with *Drosophila* may have caused the organism to be damaged. In a study with *M. domestica*, it was found that the number of eggs laid and the rate of hatching decreased in females developing in medium containing oats, milk powder, and water. Individuals of *M. domestica* that developed on a diet enriched with a mixture of milk and yeast had levels of egg production three times lower than those fed yeast and six times lower than those fed sugar (Pastor et al. 2011). It has also been shown that protein-containing food in *M. domestica* inhibits ovulation and causes a dramatic decrease in lifespan (Glaser 1923). Conversely, it was reported that addition of a protein supplement to adult housefly diet resulted in an increase in egg production (Turner and Hair 1967). In addition, the abnormal phenotypes observed in both the experimental and control groups are the result of mutations

Table 3. Effect of some foods on the sex ratio in the F_1 generation

Groups	Females	Males	Z value in-groups	Z value for males	Z value for females
Milk	650	667	G1-G5 –0.662	G5-G6 3.295*	G1-G2 –3.295*
	(G1)	(G5)		G5-G7 –4.211*	G1-G3 4.211*
				G5-G8 2.520*	G1-G4 2.520**
Yogurt	581	770	G2-G6 –7.344*	G6-G7 –7.591*	G2-G3 7.591*
	(G2)	(G6)		G6-G8 –1.287	G2-G4 1.287
Kefir	773	572	G3-G7 7.838	G7-G8 7.473*	G3-G4 –7.473*
	(G3)	(G7)			
Control	1228	1493	G4-G8 –7.218*		
	(G4)	(G8)			

*$P < 0.01$; **$P < 0.05$.

Table 4. Effect of some foods on the sex ratio in the F_2 generation

Groups	Females	Males	Z value in-groups	Z value for males	Z value for females
Milk	785	841	G1-G5 –1.965*	G5-G6 1.850	G1-G2 –1.850
	(G1)	(G5)		G5-G7 –5.554*	G1-G3 5.554*
				G5-G8 0.904	G1-G4 -0.904
Yogurt	903	1095	G2-G6 –6.102*	G6-G7 –7.725*	G2-G3 7.725*
	(G2)	(G6)		G6-G8 –1.092	G2-G4 1.092
Kefir	964	701	G3-G7 9.231	G7-G8 7.033*	G3-G4 –7.033*
	(G3)	(G7)			
Control	1154	1310	G4-G8 –4.453*		
	(G4)	(G8)			

*$P < 0.01$.

naturally occurring in *D. melanogaster*. There are individuals with abnormal phenotypes in any *Drosophila* stock. The varieties of these mutants and their chromosome locations have been determined (Lindsley and Zimm 1992). It can be concluded that the substances added to the medium do not increase the rate of these mutations. However, in toxic drug applications to *Drosophila*, these mutations increase and the rate of abnormal phenotypes also increases (Karataş and Bahçeci 2009b, Karataş and Bahçeci 2010, Karataş et al. 2011a, Karataş et al. 2012). Hence, it appears that kefir, yogurt, and milk negatively affect development but do not increase the rate of mutation causing the increase in abnormal phenotypes.

In another study, performed in humans, it was stated that a high amount of milk consumption was associated with the development of diabetes and cardiovascular diseases. Higher mortality rates were observed in males and females who consumed milk at a high rate, as well as a higher rate of fractures in females. In addition, there was a positive correlation between high consumption of milk and oxidative stress and inflammation markers and it could be associated with a higher mortality rate (Michaëlsson et al. 2014). It has been reported that milk has high d-galactose content (Song et al. 1999, Cui et al. 2004) and that d-galactose accelerates aging in different animal models. In *D. melanogaster* and *M. domestica*, the effects of d-galactose on lifespan and oxidative stress were investigated and it was found to decrease mean and maximum lifespan. This reduction in lifespan was shown to be associated with oxidative stress. Chronic exposure to d-galactose has been shown to be detrimental to health and that the addition of d-galactose via injections or diet promotes aging (Song et al. 1999, Cui et al. 2004). Even low doses of d-galactose have been seen to result in changes similar to natural aging in animals, such as oxidative stress damage, chronic inflammation, neurodegeneration, decreased immunoreactivity, and shortened lifespan due to gene-transcriptional changes (Cui et al. 2004, Cui et al. 2006, Hao et al. 2014). The reduction in the number of individuals in the

present study may be related to the d-galactose found in milk and fermented derivatives and the resulting oxidative stress.

Another reason explaining the decreasing number of individuals may be the degradation of the functioning of the basic food of *Drosophila* by alcohol fermentation (Hernández-Tobías et al. 2011) due to the nutrients. For larval and adult development in *Drosophila*, the environment in which the microorganisms involved in fruit decay are found is ideal and the organism prefers a high alcohol content/low pH environment associated with fermentative metabolism (Pohl et al. 2012, Piper 2017). It has been known for a long time that ethanol stimulates oviposition in *D. melanogaster* (Sumethasorn and Turner 2016). In this case, the function and activity of alcohol fermentation in the basic food of *Drosophila* may deteriorate when another fermented product is added to the medium, and the ethanol concentration in the medium may fall. Considering the ability of ethanol to stimulate oviposition, reduced ethanol content due to the degraded ethanol fermentation process may have caused the females to lay fewer eggs.

In the present study, in both generations, the sex ratio in the experimental group fed kefir changed in favor of females, whereas in the experimental groups fed milk and yogurt, it changed in favor of males. This shows that males are more sensitive to kefir and females are more sensitive to milk and yogurt. Indeed, there are a number of studies showing that different substances have different effects on *Drosophila* in females and males, affecting female or male development in different ways (Alentorn 1987, Davey et al. 1989, Karataş and Bahçeci 2009a, Karataş et al. 2011ab, Keser and Karataş 2012). It has been stated that protein and carbohydrates in *Drosophila* have different effects on the reproductive characteristics of the sexes and that this is due to the difference between the sexes in terms of reproductive strategies. It has been reported that during the first 12 d of adulthood, a protein increase is likely to provide an advantage in sperm competition for males due to an increase in sperm production

Table 5. Comparison of the number of abnormal individuals in the F_1 generation according to some foods

Groups	No. of abnormal individuals	Percentage of abnormal individuals (%)	Total	Between groups	Z value
Milk	21(G1)	1.59	1317	G1-G4	0.893
				G1-G3	−0.549
Yogurt	38(G2)	2.81	1351	G2-G4	−1.582
				G2-G1	2.148**
Kefir	18(G3)	1.33	1345	G3-G4	1.569
				G3-G2	−2.689*
Control	54(G4)	1.98	2721	G4-G8	

*$P < 0.01$; **$P < 0.05$.

Table 6. Comparison of the number of abnormal individuals in the F_2 generation according to some foods

Groups	No. of abnormal individuals	Percentage of abnormal individuals (%)	Total	Between groups	Z value
Milk	37(G1)	2.27	1626	G1-G4	−1.356
				G1-G3	−0.960
Yogurt	33(G2)	1.65	1998	G2-G4	0.032
				G2-G1	−1.335
Kefir	30(G3)	1.80	1665	G3-G4	−0.331
				G3-G2	0.346
Control	41(G4)	1.66	2464		

The difference was nonsignificant in all group comparisons.

(Jensen et al. 2015). In the present study, the different foods may have had different effects on the sexes for a similar reason. Alternatively, there may be recessive mutations responsible for the different rates of development with different media in males and females that are linked to sex (Hepburn et al. 2003). On the other hand, in the control group, the sex ratio was higher in favor of males, which may have been due to a problem with the medium. However, there was no visible problem with the medium during the experiments, and the reason for such a difference in this group could not be explained.

Overall, the results showed that whether milk and milk products were fermented did not change the effect on *Drosophila*, whereas they caused a decrease in the number of adults, and, contrary to expectations, impeded development, and changed the sex ratio. Considering all these results and the other studies mentioned above, it would be beneficial to investigate kefir, yogurt, and milk in other experimental organisms and in vivo. In addition, it may be useful to investigate whether kefir, yogurt, and the components of milk are potential insecticides that will not harm humans because of the high rate of development arrest shown by these test results.

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