Investigating the effect of lactic-acid bacteria on ethanol-treated *Caenorhabditis elegans*

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Abstract:

Ethanol, a depressant found in alcoholic beverages, is known to increase depressive symptoms in living organisms as it increases kynurenine levels. Ethanol also lowers the survival rate of *Caenorhabditis elegans*, an organism which has 83% of its genes which match that of humans and a nervous system similar to that of mammals, making it a good model organism for this experiment. Antidepressants are known to be ineffective on patients with heavy alcohol use. Therefore, an alternative - using *Lactobacillus* to reverse the effects of alcohol on *C. elegans* was proposed in this study. This study aimed to investigate the effect of varying ethanol volumes on the survival of *C. elegans*, and to determine if these effects of ethanol can be reversed in the presence of *Lactobacillus*. In the experiment, *C. elegans* were fed with *L. plantarum* or *L. rhamnosus* in the test setup or *E. coli* OP50 in the control setup before being exposed to varying volumes of ethanol or water as a control. We found there was no correlation between the volume of ethanol and survival rate of *C. elegans*. However, *Lactobacillus* spp. was able to increase the survival rate of *C. elegans* exposed to ethanol by an average of 43%. This suggests that *Lactobacillus* is able to reverse the effects of alcohol on *C. elegans*. Our findings thus suggest the potential use of medicine containing *Lactobacillus* as a treatment to reduce depressive symptoms in patients with alcohol-use disorders.

Introduction

Ethanol is a psychoactive drug which is found in many types of alcoholic beverages. Ethanol is known to have intoxicating effects (Chastain, 2010). World Health Organisation (2018), reports that 5.3% of all deaths worldwide are caused by excessive alcohol consumption. Alcohol-use Disorder is a brain disease, resulting in one having a negative emotional state when not consuming it (National Institute on Alcohol Abuse and Alcoholism [NIAAA], 2020). There is a causal relationship between harmful alcohol use and a range of mental and behavioural disorders, noncommunicable conditions and injuries (World Health Organisation, 2018)

Alcohol-use Disorder is comorbid with Major Depression (Lynskey, 1998; Sullivan, Fiellin, & O'Connor, 2005). One third of people with major depression have alcohol problems. (Watkins, 2020). In a study by Neupane, Lien, Martinez, Hestad & Bramness (2015), patients with higher mean scores in an Alcohol Use Disorder Identification test had higher mean serum levels of kynurenine. Kynurenine had been identified as a metabolite associated with depression (Agudelo et al., 2014). This suggests a link between ethanol intake and major depression. Watkins (2020) reported people with depression are more likely to think about suicide and high alcohol use makes antidepressants ineffective.

Lactic acid bacteria can reduce kynurenine levels. Marin et al., (2017) observed that mice with increased symptoms of despair had reduced levels of *Lactobacillus* in the gut and higher circulating levels of kynurenine. The levels of kynurenine in stressed mice can be reduced by feeding them *Lactobacillus. Lactobacillus* was found to produce H_2O_2, hydrogen peroxide, which decreases the levels of kynurenine and depression symptoms.

The *C. elegans* nervous system is similar to that of mammals (Zhu, Zhang & Li, 2014; Engleman, Katner & Neal-Beliveau, 2016). *C. elegans* is a good model organism as 83% of its genes match with humans (Lai, Chou, Ch'ang, Liu, & Lin, 2000). Upon exposure to ethanol, *C. elegans* will exhibit acute behavioural responses quantified by decrease in survival rate (Zhu et al., 2014; McIntire, 2010; de Bono & Maricq, 2005), making *C. elegans* a suitable model organism for testing the effect of *Lactobacillus* on ethanol treated *C. elegans.*

Objectives and Hypotheses

This study aimed to investigate the effect of varying volumes of ethanol on the survival of *C. elegans*, and to determine if these effects caused by ethanol can be reversed in the presence of *Lactobacillus*, hence restoring the survival rate of *C. elegans* exposed to ethanol.

It was hypothesized that an increase in the volume of ethanol would decrease the survival rate of *C. elegans*. It was also hypothesized that the survival rate of *C. elegans* fed *Lactobacillus* spp. is restored in the presence of ethanol.

Materials and Methods

Growth of precultures of microorganisms

Escherichia coli OP50 was inoculated into 10 ml of LB (Luria-Bertani) broth. *Lactobacillus plantarum* ATCC 8014 and *Lactobacillus rhamnosus* ATCC 7469 were inoculated separately in 10 ml of MRS broth. The cultures were grown overnight at 30°C in a shaking incubator. The absorbance of the precultures at 600 nm was measured using a UV-vis spectrophotometer and were standardised at 0.8.

Preparation of Nematode Growth Medium

0.9g NaCl, 7.5g agar and 0.75g bacto-peptone were added with 291.6 ml water. After autoclaving at 15 psi for 15 minutes, 0.3 ml of cholesterol (5 mg/ml), 0.3 ml of $MgSO_4$ solution (1 M), 0.3 ml $CaCl_2$ solution (1 M), 7.5 ml of KH_2PO_4 solution pH 6.0 (1M) were added.

Caenorhabditis elegans survival and locomotion assay

C. elegans N2 nematode agar blocks were obtained from stock cultures and transferred onto NGM plates and grown for 24 h at 25°C. 0.05 ml of *E. coli* OP50 preculture or 0.05 ml lactic acid bacteria culture was then added to NGM plates and grown for 24 h overnight at 30°C, as food sources for nematodes. A block of agar containing *C. elegans* N2 was then placed on the center of the NGM plate (previously added with bacteria) and incubated at 25°C for another 24 hours.

C. elegans N2 and *E. coli* OP50 or *Lactobacillus* was collected in an M9 buffer and filtered through a sterile 8 µm membrane filter. The residue (*C. elegans*), was resuspended in the M9 buffer. The M9 buffer, containing either *E. coli* OP50 or *Lactobacillus* was added to a NGM plate with either 100 µl of ethanol (100-500 mM) spread on it for the test setup, or 100 µl of sterile water for the negative control setup. The plates were incubated for 24 hours at 25°C.

Five replicates of each of the following setups were prepared for each assay:

C. elegans with *Lactobacillus* as food source on NGM agar with ethanol (test)

C. elegans with *Lactobacillus* as food source on NGM agar with sterile water (control 1)

C. elegans with *E. coli* OP50 as food source on NGM agar added with ethanol (control 2)

C. elegans with *E. coli* OP50 as food source on NGM agar added with sterile water (control 3)

The control setup was to ensure that changes in behaviour of ethanol-treated *C. elegans* is solely due to *Lactobacillus*, not *E. coli* OP50.

Variables such as amount of food, temperature and number of *C. elegans* was kept constant. The percentage survival of *C. elegans* was recorded using a microscope.

Results and Discussion

The mean percentage survival was calculated for 5 plates of the same setup with error bars plotted. Kruskal-Wallis test was conducted to test for significant difference in the results

Figure 1 :

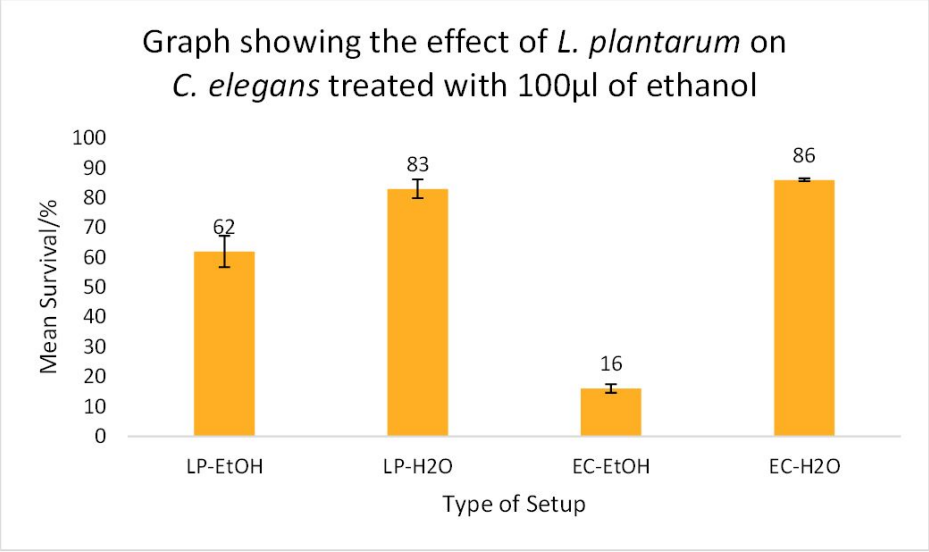

Figure 1: Graph of survival of *C. elegans* fed with *Lactobacillus plantarum* treated with 100µl of ethanol. Legend: LP - *L. plantarum*, EC - *E. coli* OP50, EtOH - Ethanol, H2O - Water

A p value of 0.00148 was obtained from Kruskal-Wallis test, showing that the mean difference between the setups was statistically significant.

Figure 2:

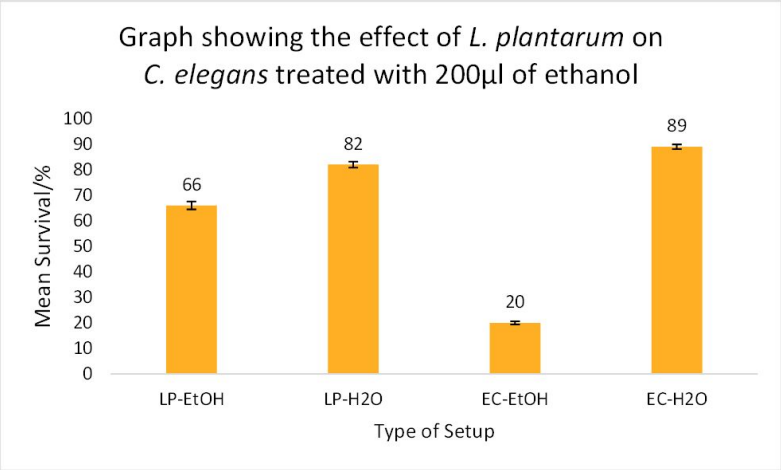

Figure 2: Graph of survival of *C. elegans* fed with *Lactobacillus plantarum* treated with 200µl of ethanol. Legend: LP - *L. plantarum*, EC - *E. coli* OP50, EtOH - Ethanol, H2O - Water

A p value of 0.00047 was obtained from Kruskal-Wallis test, showing that the mean difference between the setups was statistically significant.

Figure 3:

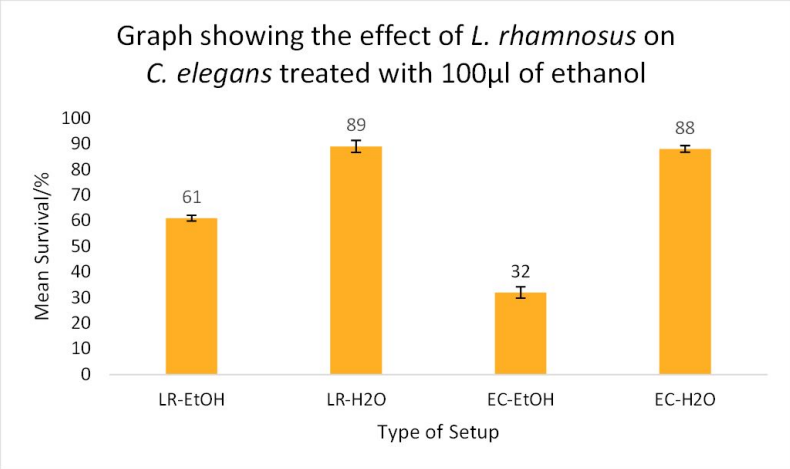

Figure 3: Graph of survival of *C. elegans* fed with *Lactobacillus rhamnosus* treated with 100µl of ethanol. Legend: LR - *L. rhamnosus*, EC - *E. coli* OP50, EtOH - Ethanol, H2O - Water

A p value of 0.00101 was obtained from Kruskal-Wallis test, showing that the mean difference between the setups was statistically significant.

Figure 4:

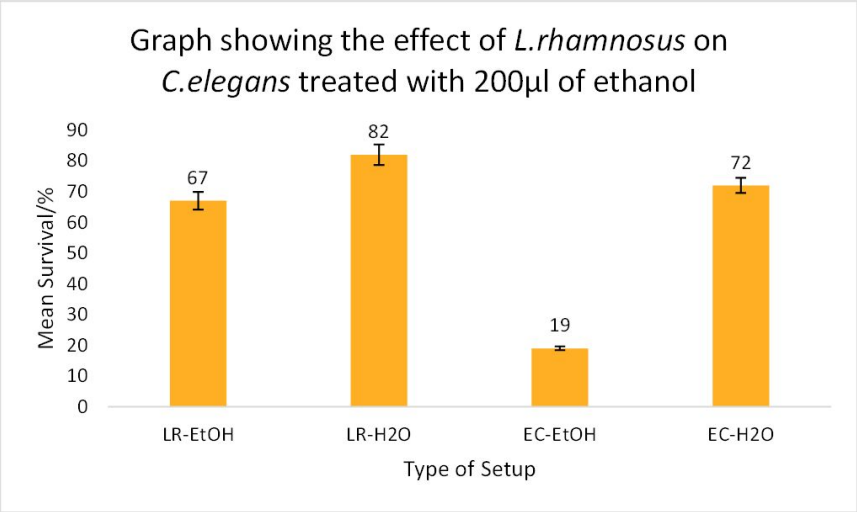

Figure 4: Graph of survival of *C. elegans* fed with *Lactobacillus rhamnosus* treated with 200µl of ethanol. Legend: LR - *L. rhamnosus*, EC - *E. coli* OP50, EtOH - Ethanol, H2O - Water

A p value of 0.00262 was obtained from Kruskal-Wallis test, showing that the mean difference between the setups was statistically significant.

Table 1: Table showing the effects of the volume of ethanol/µl on the mean survival of *C. elegans* fed with *E. coli* OP50 exposed to ethanol/%

Volume of ethanol / µl	Mean survival of *C. elegans* fed with *E. coli* OP50 exposed to ethanol / %
100	24
200	20

Table 2: Table showing the effects of the volume of water/µl on the mean survival of *C. elegans* fed with *E. coli* OP50 exposed to water/%

Volume of water / µl	Mean survival of *C. elegans* fed with *E. coli* OP50 exposed to water / %
100	87
200	85

In the control setups where *C. elegans* were fed with *E. coli* OP50 and exposed to ethanol, there was no general trend observed in percentage survival. As such, there is no correlation between ethanol volume and survival rate of *C. elegans* in the control setups

However, exposure to ethanol when fed with *E. coli* OP50 caused a significant decrease in survival rates of *C. elegans* when compared to *C. elegans* fed with *E. coli* OP50 exposed to water. When exposed to water, the mean survival rates in 100µl and 200µl of water were 87% and 85% respectively and decreased to 24% and 20% respectively in 100µl and 200µl of ethanol. This shows that ethanol has a depressive effect and results in a higher death rate on *C. elegans*. The results are shown in Tables 1 and 2.

Table 3: Table showing the effect of *L. plantarum* on survival rate of *C. elegans*/% exposed to various volumes of ethanol/µl

Setup	% survival when fed with *L. plantarum* / %	% survival when fed with *E. coli* OP50 / %	Survival Rate increase/ %
100µl ethanol	62	16	46
200µl ethanol	66	20	46

Table 4: Table showing the effect of *L. rhamnosus* on the survival rate of *C. elegans*/% exposed to various volumes of ethanol/µl

Setup	% survival when fed with *L. rhamnosus* / %	% survival when fed with *E. coli* OP50 /%	Survival Rate increase / %
100µl ethanol	61	32	29
200µl ethanol	67	19	48

L. plantarum had increased the survival rate by an average of 46% across varying volumes of ethanol while *L. rhamnosus* had increased the survival rate by 39% across varying volumes of ethanol. There was a consistent trend of reversal of *Lactobacillus* reversing the effects of ethanol on *C. elegans*. *L. plantarum* and *L. rhamnosus* increased the survival rate of *C. elegans* exposed to ethanol by an average of 43%, showing a reversal in effects of exposure to ethanol. The results are shown in Tables 3 and 4.

Exposure to ethanol decreased the survival rate of *C. elegans* as it likely has a depressive effect on *C. elegans*. *Lactobacillus spp.* This matches literature which shows that alcohol consumption is comorbid with major depression (Lynskey, 1998; Sullivan, Fiellin, & O'Connor, 2005). *Lactobacillus* increased the survival of *C. elegans* exposed to ethanol as it may have increased immune defence and stress resistance, matching that of literature (Komura, Ikeda, Hoshino, Sibamura & Nishikawa, 2012). Our findings are similar to literature by Marin et al., (2017), which shows *Lactobacillus* reduces depression symptoms in mice. *C. elegans* is a good model organism as 83% of its genes match with humans (Lai, Chou, Ch'ang, Liu, & Lin, 2000). There have been few research papers which use *C. elegans* as a model organism in our area of study.

Conclusion and future work

Our study had shown there is no correlation between the volume of ethanol and survival rate of *C. elegans.* At the same time, we concluded the rate of survival of *C. elegans* fed *Lactobacillus* is increased by an average of 43 percent in the presence of ethanol.

One of the major limitations in our study was the inability to consistently standardise the lifespans, sizes and quantity of the *C. elegans* present throughout all the different set-ups. The stages of growth of *C. elegans* could not be controlled.

An application of the results of our research is its potential use in the pharmaceutical industry. *Lactobacillus* may be used in medical therapy and an alternative to antidepressants for alcohol use disorder patients to reduce symptoms of depression.

A possible extension to our research is that different strains of *Lactobacillus* with known benefits to humans such as *L. casei*, *L. acidophilus* can be used in experiments to test for similar effects. At the same time, the incubation period can be increased from 24 hours to 48 hours to test if the *Lactobacillus* has a sustained ability on reversing the effect of ethanol on *C. elegans* or delays the effects of ethanol. More quantitative data such as number of body bends and locomotion could be obtained to improve our understanding of the effect of *Lactobacillus* on *C. elegans* treated with ethanol.

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