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Attenuating free radical damage with fermented Geoje yuzu in *Drosophila*

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ABSTRACT

Introduction: Yuzu, a yellow citrus fruit from a plant belonging to the *Rutaceae* family, is a popular ingredient in many types of processed beverages. However, the long-term consequences of yuzu consumption at the organism level have not been thoroughly studied yet. Post-harvesting methods and cultivation methods may influence the reported health-promoting properties of yuzu, including antioxidant, anti-inflammatory, and anti-cancer effects. Therefore, we chose *Drosophila* as an animal model to evaluate the beneficial effects of yuzu that is cultivated and processed through fermentation in Geoje, South Korea. We examined *in vivo* effects of fermented yuzu on *Drosophila* during its lifetime. **Methods:** Using lifespan assay, flies were fed fermented Geoje yuzu (FGY) and their survival was monitored. In healthspan assays, flies fed with FGY were exposed to environmental stresses, such as starvation, hydrogen peroxide, or carbon dioxide (CO_2). Effects of FGY on improving ability of flies to withstand these stresses were assayed. **Results:** Consumption of yuzu did not increase lifespan of the flies nor their capacity to withstand starvation and CO_2 stress. However, it significantly protected the flies from oxidative stress, as evidenced by an increase in the survival rate of flies fed a diet supplemented with FGY when exposed to hydrogen peroxide. **Conclusion:** This research found that FGY protects flies from oxidative stress. These results may pave the way for future research on long-term health-promoting properties of yuzu, ultimately fostering collective efforts to maximise health benefits of yuzu.

Keywords: *Drosophila*, fermentation, oxidative stress, yuzu

INTRODUCTION

Yuzu (*Citrus junos*) is a yellow fruit with an uneven, thick skin, measuring approximately 4 to 7 centimetres in diameter. It is obtained from the citron tree that belongs to the *Rutaceae* family and is classified within the *Sapindales* order. The citron tree is believed to originate from China and was introduced to Korea and Japan around the 8th century (Swingle, 1967). Today, it is consumed predominantly as an additive in a variety of foods and also in the form of tea. The unique flavour profile of yuzu, a combination of bitterness and astringency with a hint of sourness, is one of the main reasons for its widespread consumption. These flavours contrast with those of tangerines and oranges, belonging to

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the same family. From a nutritional point of view, it contains a variety of bioactive substances, including vitamin C and flavonoids, such as hesperidin, which have been demonstrated to possess antioxidant, antimicrobial, and anti-inflammatory properties, as well as retaining the potential to inhibit the proliferation of cancer cells (Hirota *et al.*, 2010; Kim *et al.*, 2014; Lee, Hyun & Jung, 2020).

As with other fruits and grains obtained from nature, climatic conditions and methods of cultivation influence the composition of bioactive components of yuzu. Tu Youyou, the 2015 Nobel Laureate in Physiology or Medicine known for her discovery of the antimalarial drug artemisinin derived from sweet wormwood, observed that post-harvesting procedures, including the extraction of bioactive compounds from natural products, can significantly affect the composition of biologically active elements (Tu, 2011). Consumer preference is inevitably influenced by the difference in the composition of bioactive components. For example, it has been reported that consumer preference for yuzu has increased with the reduction of its astringent, gritty taste due to the use of modern food preparation techniques in the production of standardised yuzu groundwater (Park, Oh & Cho, 2019). However, there is a dearth of studies to investigate the health-promoting effects of yuzu cultivated in a particular region and subject to a distinctive post-harvesting protocol.

Several reports indicated that yuzu may have the potential to cause toxicity. It has been documented that yuzu can induce anaphylactic reactions, such as food-dependent or exercise-induced anaphylaxis (Tanaka *et al.*, 2017). It appears that citron consumption may exert a direct or indirect influence on the pathogenesis of various diseases of the cardiovascular system, such as the inhibition of platelet aggregation and the prolongation of blood clotting time (Yu *et al.*, 2011). Due to the current mode of consuming yuzu as food and an ingredient in various beverages, it is important to study the effects of long-term consumption of yuzu. However, there is a paucity of studies that use *in vivo* animal models to assess the effects of long-term consumption of yuzu.

At the organismal level, the fruit fly is one of the most commonly used experimental animal models to monitor the consequences of long-term drug or food administration that span the lifetime of the organism. The fruit fly is an attractive *in vivo* animal model to address a range of scientific questions because of its relatively short lifespan (approximately two to three months) and the availability of potent yet cost-effective genetic manipulation tools. Its track record of recapitulating human diseases to a sufficient extent to examine crucial biological hypotheses has further cemented its position as a valuable research tool. *Drosophila* has been used to study mechanisms of ageing, neurological diseases, hepatic disorders, and cancers (Victor Atoki *et al.*, 2025).

Hence, in this study, the long-term health benefits of fermented yuzu that is cultivated particularly in Geoje, the southernmost part of the Korean peninsula, were assessed. Due to globalised climate change, this region is undergoing a rapid transformation to become a centre for the cultivation of subtropical plants, such as geuk, chayote, and passion fruit. To study the health benefits of yuzu, fruit flies were employed as an experimental model organism. This research hopes to facilitate the collective efforts in understanding the health benefits of yuzu.

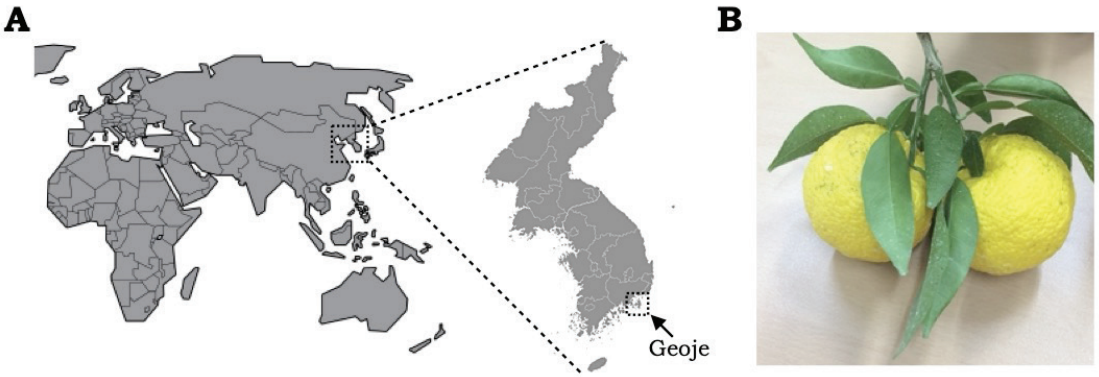

Figure 1. Photo of yuzu planted and grown in Geoje, South Korea. A. The dotted square indicates the location of Geoje Island in South Korea. **B.** Photo of yuzu used for fermentation in the experiments.

MATERIALS AND METHODS

Preparation of fermented Geoje yuzu

Yuzu fruits harvested in 2019 from trees of 35 to 40 years of age and cultivated in Sadeung-myeon, Geoje, South Korea, were used (Figure 1). They were washed with water. Entire fruits, including peels, were crushed using a crusher. Ten kilos of crushed yuzu were combined with 12 kilos of sugar. The resulting mixture was then placed in clay jars, covered with lids, and aged at 4°C for a period of six months to allow for fermentation. After six months, the fermented yuzu mixture was filtered through a sieve with a diameter of approximately 500 to 750 μm. The resulting raw solution was designated as fermented Geoje yuzu (FGY). FGY was aliquoted into 50 mL Falcon tubes and stored at -20°C until needed for subsequent experiments. All experiments involving FGY were completed by November 2020.

Fly husbandry, diet preparation, and crossing

w^{1118} strain flies were employed, maintained, and handled, as described in our previous studies (Kim, Kim & Kim, 2021; Niraula & Kim, 2019). Briefly, the flies were housed in *Drosophila* bottles made of polypropylene (size: 57 × 57 × (H) 103 mm). They were fed a standard diet that was prepared by initially mixing 8.5 g cornmeal, 7.5 g sugar, 1.5 g live yeast, and 0.46 g agar in 100 mL distilled water, followed by brief boiling. The mixture was left to cool down at room temperature. Once the temperature dropped to around 80°C, a final 1% v/v concentration of acid mix (a mixture of propionic acid, phosphoric acid, and water at a ratio of 41.80:4.15:54.05) and 10% tegosept dissolved in 95% ethanol (EtOH) was added to the mixture to prevent the growth of mould. The flies were kept in an incubator controlled for light (12 hours light/dark cycle), temperature (25°C), and humidity (60%). Unless otherwise specified, all experiments were performed in the fly incubator. Approximately every three weeks, flies were transferred to fresh bottles to refresh the stock.

For crossing, ten virgin female flies were kept together with three to five male flies inside the *Drosophila* bottles containing 50 mL of standard diet for four days. The parent flies were then discarded, while the larvae in the bottles were left to eclose. The flies were sorted out 14 days after crossing. Carbon dioxide (CO_2) was used to temporarily anaesthetise flies for sorting. The sorting

was completed within three minutes to minimise CO_2 toxicity. Sorted flies were fed on an *ad libitum* diet (AL) or an AL-supplemented diet with a 1% or 15% concentration of FGY (hereafter 1% FGY or 15% FGY). The recipes for the preparation of AL were the same as the standard diet, with the exception that 1.5 g live yeast and 7.5 g sugar were replaced with 5 g yeast extract and 5 g sugar, respectively. Likewise, the recipe for 1% or 15% FGY was the same as the AL diet, with the exception that a 1% or 15% v/v concentration of FGY was added to the AL media before it was boiled. Experiments using *Drosophila* do not require approval from Inje University's Institutional Animal Care and Use Committee (IACUC).

Lifespan, starvation, and hydrogen peroxide (HP) stress assays

Sorted adult flies were reared in long acrylic fly vials (φ 30 × 180 mm) containing AL with or without FGY (1% or 15%). For lifespan assays, the survival of flies was scored when transferred to freshly made AL or AL supplemented with FGY every Monday, Wednesday, and Friday. Only mated adult flies were used throughout the experiments since it has been shown that mated female flies, in particular, are shorter-lived than virgin female flies (Flatt, 2011). Therefore, freshly eclosed male and female flies were allowed to stay together for an additional four days prior to sorting to provide them sufficient time to mate. Moreover, only mated female flies that did not have virginity markers, such as the wet appearance of wings and body and the presence of meconium (a dark green area on the abdomen), which represents the remains of larval food, were collected.

Healthspan is defined as the length of time a person is expected to live in a state of good health. It is calculated by subtracting the expected duration of periods of incapacitation due to illness or accident from the duration of lifespan. A common measure of healthspan is the capacity to withstand stressful stimuli. Hence, the effects of FGY on the healthspan of flies were assessed during starvation, free radical stress assays, and recovery time following anaesthesia with CO_2.

In the starvation assay, the ability of flies to mobilise their stored food under nutritional stress without becoming dehydrated was examined. Adult fruit flies were separated into two groups: one that was fed FGY and one that was not. The flies were maintained for ten days. The period of ten days was selected since it is a considerable length of time in the context of a fruit fly's lifespan. We hypothesised that this period of ten days would be sufficient to discern whether the ingestion of FGY would alter the physiology of fruit flies. Following ten days of FGY consumption, the fruit flies were transferred to an agar medium comprising solely of water and minerals. The survival time was subsequently measured every four hours (Figure 3A). Hydrogen peroxide, known to induce free radical reactions in living organisms including *Drosophila,* was used in the free radical stress assay (Zhang *et al.*, 2023). Hereafter, free radical stress assay will be referred to as HP stress assay. For the HP stress assay, the sorted flies were fed on AL or AL supplemented with 1% FGY for ten days, then exposed to 0.3% hydrogen peroxide. Survival curves were generated after scoring dead flies every day (Figure 5A). Flies were transferred to fresh starvation or exposed to hydrogen peroxide with or without FGY-supplemented media each day, and the dead flies were eliminated when possible.

Anaesthesia recovery time (ART) experiment

CO_2 is a commonly utilised anaesthetic for fruit flies. However, it is known to induce

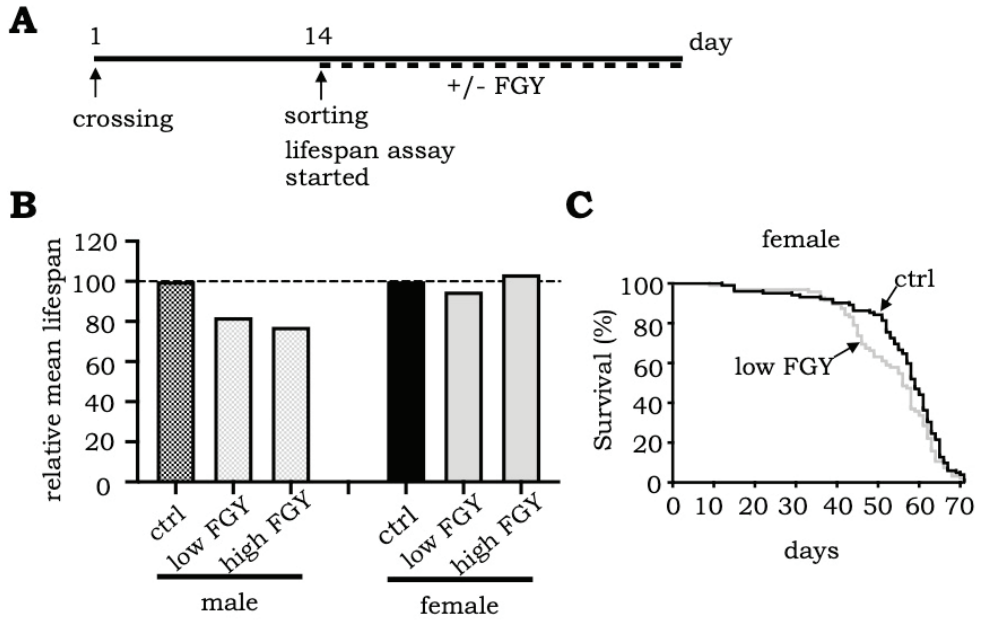

Figure 2. FGY does not increase lifespan in Drosophila. A. A schematic diagram depicting the lifespan assay protocol. **B.** In comparison with the lifespan of the control files (fed with AL), the relative mean lifespan of flies fed with low FGY (1%) or high FGY (15%) was calculated. **C.** Representative lifespan curves of female flies fed a diet supplemented with or without (ctrl) low FGY (1 %). *n* = 25-102 for each group.
FGY: fermented Geoje yuzu; AL: *ad-libitum*; ctrl: control

significant stress responses, including acidification of the haemolymph, reduction in heart rate, and inhibition of adenosine triphosphate (ATP) production due to oxygen deprivation (Colinet & Renault, 2012). Fruit flies exposed to CO_2 exhibit decreased fertility and impaired motor performance compared to that observed in the non-exposed control group (Barron, 2000; Bartholomew *et al.*, 2015). Therefore, this study examined if consumption of FGY could help to overcome this stress. The adult fruit flies were maintained on AL for ten days, with the control group receiving AL and the experimental group receiving AL supplemented with 1% FGY. Following this, both groups were exposed to 100% CO_2 for three minutes to induce complete anaesthesia (Figure 4A). The time required for the flies to regain consciousness was then measured following the removal of CO_2, and a time curve for the recovery process from anaesthesia was generated.

Reagents

Drosophila diet ingredients (cornmeal, sugar, live yeast, yeast extract, and agar) were purchased from Hansol Tech Inc. (Seoul, Korea). Propionic acid (cat# 64655-0430) was obtained from Junsei Chemical Co. Ltd. (Tokyo, Japan). Tegosept (cat# H3647) and phosphoric acid (cat# 345245) were purchased from Sigma-Aldrich (St. Luis, USA).

Statistics

For the lifespan, starvation, HP stress assays, and ART experiment, Prism 8 (Graphpad, USA) was used to analyse the data and log-rank test was used to determine statistical significance.

RESULTS

Effect of FGY on the lifespan of flies

To examine the effects of long-term consumption of FGY on the lifespan of organisms, adult fruit flies were fed a diet containing either a low (1%) or high (15%) concentration of FGY. However, as illustrated in Figure 2, neither concentration increased the lifespan of fruit flies. Although it is possible that higher concentrations of FGY could prolong lifespan, a concentration of 15% is reasonably high when considered in the context of typical yuzu intake practices in everyday life. A fly typically consumes approximately 1 μL of food per day (Carvalho, Kapahi & Benzer, 2005). Therefore, when given 15% FGY, the amount of FGY a fly would consume per day is approximately 0.15 μL. Considering the weight difference between a fly (1 mg) and a human (70 kg), the consumption of 15% FGY by a fly could be equivalent to the consumption of 10.5 L of FGY by a human each day. Therefore, it was deemed unnecessary to increase the concentration of FGY beyond this level. Although prolonged consumption of FGY did not increase the lifespan of fruit flies, other experiments were conducted to explore other *in vivo* physiological activities of FGY. Since low and high concentrations of FGY did not differ in their effects to increase the lifespan of fruit flies, subsequent experiments were conducted using low concentrations of FGY.

Effect of FGY on modifying the capability of flies to withstand starvation

As illustrated in Figures 3B-3C, the consumption of FGY did not increase the ability of the flies to resist starvation stress. Instead, a decrease in the ability to withstand starvation stress was observed in the female group that was fed FGY.

Effect of FGY on altering the ability of flies to recover from anaesthesia

The influence of FGY on the ability of

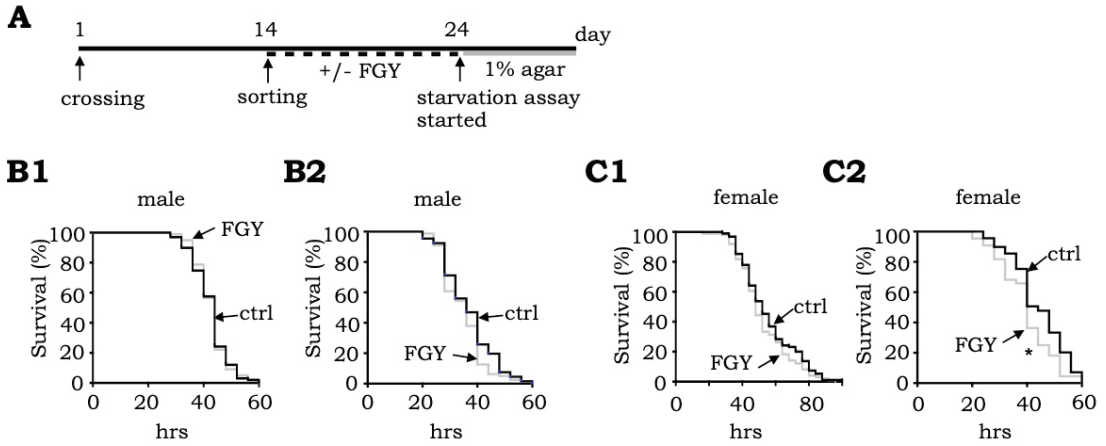

Figure 3. FGY does not protect Drosophila from starvation stress. A. A schematic diagram depicting the starvation assay protocol. **B-C.** Once sorted, male (**B1-B2,** replicates) or female (**C1-C2,** replicates) flies were fed AL (ctrl) or AL supplemented with 1% FGY for 10 days. Starvation assays were then started and dead flies were scored every four hours, from which the survival curves were generated with Prism 8.0 software. n = 44-99 for each group. *p<0.05 Log-rank test.
FGY: fermented Geoje yuzu; AL: *ad-libitum*; ctrl: control

flies to recover from stress caused by exposure to CO_2, a commonly used anaesthetic in *Drosophil*a research, was examined and depicted in Figure 4A. As demonstrated in Figures 4B-4C, the consumption of FGY did not result in faster recovery from anaesthesia.

Effect of FGY to counteract hydrogen peroxide-induced shortening of lifespan

As illustrated in Figures 5B and 5C, hydrogen peroxide hastened the death of flies. Approximately 50% of flies in the control group died following ten days of exposure to hydrogen peroxide. However, the survival rate was *higher* in the group of flies fed a diet supplemented with FGY from the sorting process.

DISCUSSION

The objective of this study was to evaluate the effects of FGY on the lifespan and healthspan of organisms. The term "lifespan" is used to indicate the length of time for which an organism is alive, whereas "healthspan" describes the number of years an organism is healthy without chronic and debilitating diseases. Despite growing research evidence stating that a treatment that prolongs lifespan also extends healthspan (Kapahi, Boulton & Kirkwood, 1999), current data challenges this long-held belief. For example, dietary restriction, a strategy widely used to extend lifespan, has been shown to be ineffective in attenuating cognitive function decline with advances

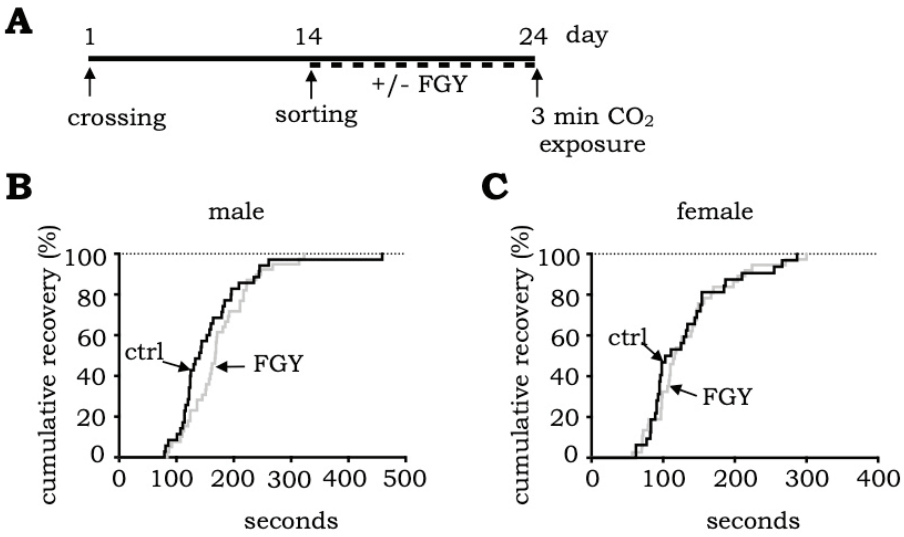

Figure 4. FGY does not accelerate the recovery of flies who have been rendered unconscious by CO_2-based anaesthesia. A. A schematic diagram depicting the CO_2-based anaesthesia recovery time (ART) experiments. **B-C.** Once sorted, male (**B**) or female (**C**) flies were fed a diet supplemented with or without 1% FGY for ten days. Flies were then exposed to CO_2 for three minutes to induce complete anaesthesia. The wake-up time of an individual fly was measured, from which the cumulative recovery curves were constructed with Prism 8.0 software. n = 32-39 for each group.
FGY: fermented Geoje yuzu; CO_2: carbon dioxide; ctrl: control

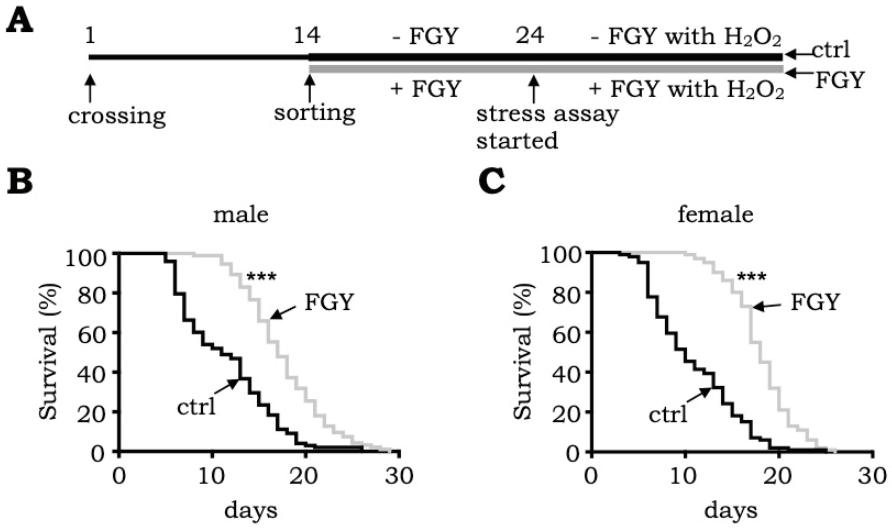

Figure 5. FGY attenuates H_2O_2-induced lifespan shortening in Drosophila. A. A schematic diagram depicting the H_2O_2 oxidative stress assay protocol. **B-C.** Once sorted, male (**B**) or female (**C**) flies were fed a diet in the presence (FGY group) or absence (control group) of 1% FGY for ten days. Control flies were then fed AL supplemented with 0.3% H_2O_2 in the absence of FGY while FGY flies were fed AL concomitantly supplemented with FGY and exposed to 0.3% H_2O_2. Dead flies were scored every day when flies were transferred to freshly made media. The survival curves were built with Prism 8.0 software. n = 94-100 for each group. ***p<0.001, Log-rank test.
FGY: fermented Geoje yuzu; H_2O_2: hydrogen peroxide; AL: *ad-libitum*; ctrl: control

in age in *Drosophila* (Burger, Buechel & Kawecki, 2010). Most notably, despite a dramatic increase in life expectancy in humans due to advances in medical science, their healthspan has not been prolonged to a similar extent (Kovacs, Brunner & Ari, 2021). In line with the above observations regarding the distinction between lifespan and healthspan, our results demonstrated that FGY promotes healthspan without affecting lifespan. Moreover, the concept of healthspan is, to some extent, an abstract one. A given substance may have a plethora of effects on bodily functions. As a result, depending on the specific region of the body under examination, a given substance may either enhance the functionality (thereby increasing healthspan) or diminish the functionality (thereby decreasing healthspan). Therefore, although we have demonstrated that FGY protects flies from oxidative stress, a reliable indicator of healthspan, we cannot exclude the possibility that it may also have adverse long-term effects on other aspects of fly physiology.

To determine whether FGY could modify the capacity of flies to withstand environmental stress, three types of stimuli were employed. Although no change in the capacity of the flies to withstand starvation and recuperate from damage caused by CO_2 anaesthetics was observed, a detailed discussion of the results may be worthwhile. Flies can withstand starvation by utilising the energy stored within their bodies. Glucose is the primary energy source. However, the capacity to survive prolonged starvation relies on the quantity of stored fat in the body prior to the onset of starvation

and the efficiency of enzymes involved in converting stored fat into ATP. Hence, two additional experiments may be required to elucidate the mechanisms underlying the ineffectiveness of FGY in resisting starvation due to stress. This would entail measuring the total amount of triglyceride and analysing the protein levels of key enzymes involved in fat metabolism, such as Acetyl-CoA-Carboxylase (ACC) (Katewa *et al.*, 2012). Another noteworthy observation in the starvation assay was that in the presence of FGY, female flies demonstrated a slight reduction in their ability to resist starvation stress (Figure 3C2). This finding suggests that the effects of FGY are sex-specific. While the precise mechanisms remain unclear, it is well established that there are numerous sex-specific diseases. For example, many autoimmune diseases, such as Graves' disease and Hashimoto's thyroiditis, are more prevalent in females. Conversely, men tend to experience worse outcomes from COVID-19 infection (Migliore, Nicoli & Stoccoro, 2021). This indicates the potential existence of sex-specific pathophysiological processes in certain disorders. Similarly, numerous pharmaceuticals, including the sleep aid zolpidem, exhibit sex-dependent differential effects (Zhao *et al.*, 2023). Hence, it is necessary to adjust the dose or carefully monitor potential side effects when administering these agents on the basis of sex. Although it should be determined experimentally, we speculate that FGY's pharmacokinetic parameters or potential interactions with sex hormones may contribute to this intriguing phenomenon.

The quantity of agents employed in an experimental model system is important to examine their actions in experiments designed to elucidate the potential health-promoting properties of their bioactive components. Based on this rationale, two distinct doses of FGY were utilised in the lifespan assay: 1% and 15%. The 1% dose was selected for subsequent experiments based on the outcomes of the lifespan assay (Figure 2). Similarly, in the case of the CO_2 anaesthesia recovery assay, the concentration of CO_2 was taken into account. To achieve the desired anaesthetic effect, 100% CO_2 was employed. However, for this type of experiment, duration is another adjustable parameter. In this study, the flies were exposed to CO_2 for three minutes. Since previous studies reported that as little as a five-minute exposure to CO_2 can cause a deficit in motor activity, a three-minute exposure was selected to achieve complete anaesthesia while minimising neuronal damage (Bartholomew *et al.*, 2015). This time frame is widely adopted as a standard protocol for fly anaesthesia in a variety of experiments (Katewa *et al.*, 2012). However, in real-life situations, it is evident that stress levels can vary considerably, from relatively mild to severe. Given that a three-minute duration causes a 100% anaesthetic effect in all flies and is close to the time point that could potentially harm the body system, it can be classified as a quasi-maximal stress. If this assumption is valid and the protective effect of FGY is moderate, the current assay protocol may not be optimal for elucidating the subtle effects of FGY. Harsh CO_2 stress may mask FGY's potential health-promoting effects. Therefore, a milder stressor may be necessary to uncover the hidden effects of FGY.

This research demonstrated the most promising beneficial effect of FGY to alleviate the reactive oxygen species (ROS) burden in organisms, as illustrated in Figure 5. Although several studies have demonstrated yuzu's antioxidant activity, most experiments were performed using *in vitro* platforms or focused on yuzu's impact on specific cellular functions, such as maintaining

the integrity of the blood-brain barrier (Choi *et al.*, 2022; Lee *et al.*, 2020). To the best of our knowledge, this research is the first to systematically investigate yuzu's ability to protect an organism against ROS damage through its entire lifespan. As shown in Figure 5, the results demonstrated that exposing flies to hydrogen peroxide (HP) and feeding them with FGY confers protection against HP stress. Nevertheless, it is of utmost importance to exercise caution when interpreting these intriguing results. From a mechanistic standpoint, this can be achieved through FGY-mediated upregulation of the fly's ROS scavenger system. Specifically, FGY may upregulate the expression of superoxide dismutase, catalase, and/or glutathione peroxidase, all of which are responsible for eliminating the body's ROS, thereby reducing cellular damage triggered by free radicals (Ighodaro & Akinloye, 2018). Our group previously demonstrated that extracts from *Ilex paraguariensis*, commonly called yerba mate, enhance the capacity of flies to withstand ROS stress by increasing the expression levels of catalase and glutathione peroxidase (Niraula *et al.*, 2018). Therefore, it would be worthwhile to investigate whether similar changes at the cellular level can be observed when FGY is consumed.

There is an alternate interpretation of Figure 5. In the experimental protocol, the flies were simultaneously exposed to HP and fed a diet with FGY. The design of this experimental protocol was necessary because flies were dying slowly upon exposure to HP. As illustrated in Figure 5, approximately ten days were required for half of the flies to succumb to death following exposure to 0.3% HP. If the flies were fed FGY for only ten days from sorting prior to exposure to HP, the ten-day FGY effect would be gradually diminished over the course of the HP assault. Therefore, an alternate interpretation could be proposed as follows: rather than increasing the number of enzymes involved in eliminating free radicals, FGY may directly neutralise the effect of HP by chemical reactions. Consequently, the actual amount of harmful HP in AL supplemented with FGY would be significantly lower than that of AL exposed to HP alone. Therefore, the presence of FGY would render the flies less susceptible to the toxic effects of HP. To determine if this hypothesis is true, we need to directly measure changes in the levels of (1) the gene transcripts associated with the ROS scavenger system and (2) free radicals in AL supplemented with or without FGY and exposed to HP.

In our study, we used fermented yuzu instead of fresh yuzu. Metabolic consequences vary with different foods that are subject to fermentation. For fruit fermentation, lactic acid bacteria (LAB) have been shown to be a major microorganism responsible for producing biologically active substances, such as vitamins, minerals, and enzymes. These substances contribute to numerous health benefits, including but not limited to antimicrobial, anti-carcinogenic, anti-allergic, and even anti-hypertensive effects (Sanlier, Gokcen & Sezgin, 2019). Furthermore, Seoung *et al.* demonstrated that fermentation increases the production of naringinase that converts the bitter-tasting naringin found in yuzu peels to the tasteless naringenin, thus enhancing consumer acceptance of yuzu (Nara *et al.*, 2024; Seong *et al.*, 2023). Therefore, it would be useful to examine the extent to which post-harvesting methods of fermentation affect the composition of bioactive ingredients present in yuzu. Such knowledge would help to optimise post-harvesting processes and maximise the health-promoting benefits of yuzu.

Finally, given that the experiments were performed with yuzu harvested in 2019, changes in climate and cultivation practices since then may have altered the bioactive properties of yuzu. Therefore, conducting a longitudinal assay with yuzu collected at different time periods may help to understand the effects of environmental factors on yuzu's health-promoting capabilities.

CONCLUSION

Although the consumption of FGY did not affect the lifespan of flies, it protected them from free radical stress and decreased HP-induced mortality. This study provided the first evidence of the long-term effects of fermented yuzu consumption in an *in vivo* animal model, thereby facilitating further investigations aimed at optimising and fully elucidating the health benefits of yuzu.

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Authors' contributions

Kim MS, principal investigator, conceptualised, designed the study, supervised the experiment, prepared the draft of the manuscript, and reviewed the manuscript; Park K, Kim H, Park J, and Lee M set up the experiment; Park K, Kim H, and Park J led data collection, data analysis, and statistical analysis. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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