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Physiological Functions Mediated by Yuzu (*Citrus junos*) Seed-Derived Nutrients

Mayumi Minamisawa

Abstract

This section is focused on the physiological functions of yuzu (*Citrus junos*) to improve health. The modern lifestyle involves number of modern lifestyles involve various factors that may increase the production of active oxygen species. Nutritional supplements and medicines are commonly utilized to maintain health. Yuzu seeds contain >100-fold the limonoid content of grapefruit seeds and are rich in polyamines (PAs), including putrescine, spermidine, and spermine. Limonoid components mediate the antioxidant properties of citrus. Limonoids and PAs convey various bioactivities. PAs are closely associated with maintaining the function of the intestinal mucosal barrier, which might be involved in the metabolic processes of indigenous intestinal bacteria and in the health of the host. After ingestion, food is digested and absorbed in the intestinal tract, which is also responsible for immune responses against food antigens and intestinal bacteria. Detailed investigations of the physiological functions of extracted yuzu seed extracts may help to develop new treatment strategies against diseases associated with inflammatory responses.

Keywords: Yuzu (*Citrus junos*), limonoids, polyamine, gut microbiota, anti-inflammatory, short-chain fatty acid (SCFA), central neurodegenerative disease

1. Introduction

In 1997, the World Cancer Research Fund published 14 articles concerning dietary recommendations in addition to smoking cessation for the prevention of cancer in *Food, Nutrition and the Prevention of Cancer: a Global Perspective* (2007 revised edition) to promote international awareness of the relationship between nutrition, diet, and cancer. Articles 1, 4, and 5 strongly recommend the consumption of foods of plant origin, and especially emphasized the importance of fruits and vegetables for the prevention of many types of cancer [1].

There are more than 1000 species of citrus and various varieties account a major part of all fruit production worldwide. In particular, citrus species native to Asia are believed to have originated in the Assam area of India around 30–40 million years ago and were propagated in China, Thailand, Malaysia, Indonesia, and Taiwan before being brought to Japan [2]. The tachibana orange, which is the oldest variety of mandarin orange in Japan, was introduced in Japan from Taiwan via the Korean Peninsula from mainland China, and is listed in the *Manyoshu*, the oldest extant

collection of classical Japanese poetry compiled sometime after 759 AD during the Nara period, as the only citrus fruit that existed in the wild. After that, it is estimated that the daidai, an Asian variety of bitter orange, and other small oranges arrived in Japan around 2 to 300AD. Yuzu (*Citrus junos Sieb. ex Tanaka*) originated in China and was introduced to Japan and other countries around the 4th to 8th centuries, as this fruit is mentioned in the *Shyoku-Nihongi*, an imperially-commissioned Japanese history text completed in 797 AD.

The traditional Japanese meal *washoku* was recognized as a UNESCO Intangible Cultural Heritage of Humanity in 2013. The Japanese have the highest life expectancy of any other ethnicity. Therefore, *washoku* has attracted attention as a healthy diet. Especially, yuzu is an essential ingredient of the Japanese diet in the winter months. A traditional Osechi dish, including yuzu, to be eaten on New Year's Day is shown in the photo in **Figure 1**.

Yuzu is a commercially important fruit, as compared to other sour citrus fruits, and has become very popular in Japan. Although rarely eaten as a fruit, yuzu is a common ingredient in Japanese cuisine, where the aromatic zest (outer rind) as well as juice are used much in the same way as lemons in other cuisines. The yuzu fruit and juice are traditionally used in making vinegar and seasoning yuzu peel and juice, and along with sudachi, daidai, and other similar citrus fruits, are integral ingredients in the citrus-based sauce ponzu. In addition, yuzu is often used as an ingredient in alcoholic drinks, such as the yuzu sour. Recently, yuzu kosho “yuzu and pepper” has become a very popular spicy Japanese sauce made from the peel (zest) of green or yellow yuzu, combined with green or red chili peppers and salt. Yuzu is also well-known because of its pleasant aroma and essential oil of the outer rind. In fact, in Japan, it has been customary since ancient times to take a bath with yuzu in hot water during the winter solstice. The yuzu peel is particularly high in aromatic compounds and pectin; therefore, the waste peel from juice extraction is sometimes used to produce essential oils and flavorings as well as for medicinal purposes. Similarly, yuzu is industrially used in the production of sweetened beverages, cosmetics, and perfumes, as well as oils for aromatherapy [3]. Only a small portion of produce is used for natural medicine, while satsuma mandarins, oranges, and grapefruits are commonly used for the production of fruit juices.

There is a reason why yuzu is not often eaten as a fruit in Japan because it contains large seeds that convey a bitter taste to the juice. The well-known constituents of citrus fruits include essential oil components, flavonoid glycosides, and other basic substances with biological activities, including limonene, a major component of essential oils found in the juice and rind, polymethoxyflavones, coumarins, carotenoids, which are pigments, vitamins, and terpenoids [4–7]. In the past, the bitterness of citrus juices, skins, and seeds hindered the demand for citrus fruits.



Figure 1.
A typical Osechi package for New Year's day in Japan.

Much research has been conducted to produce bitter-free citrus fruits. At the time when there was very little demand for bitter fruit and juice, Hasegawa et al. [8–10] reported that the high physiological activity of limonoids was responsible for the bitter taste in citrus juice. Limonoids are a group of triterpene derivatives found in plants of the Rutaceae and Meliaceae families. So far, more than 300 types of limonoids have been reported, and about 100 types have been isolated from the neem and sendan plants of the family Meliaceae.

Limonoids are characterized by a furan ring at C-17, a lactone ring at C-3 or C-6, and an epoxide between C-14 and C-15 (**Figure 2**). Of the four basic structures of limonoids, reversible opening and closing occurs in A, D lactone rings. For example, in the case of limonin, one of the major limonoids, a closed D-ring creates a bitter taste, while the open D-ring form (limonate A-ring lactone) has no bitter taste. Furthermore, the limonate A-ring lactone at C-17 is converted into the limonoid glycoside 17- β -D-glucopyranoside, which is D-glucose bound with β -glucoside.

Until recently, not much had been known about the metabolism of limonoids found in fruit. In 1991, Hasegawa et al. [11] discovered that limonate A-ring lactone, an open D-ring form of a limonoid aglycone, was metabolized to a glucoside derivative in the late stages of fruit growth and maturation, and suggested that this occurred independently in both the seeds and fruit. It is known that the limonoid aglycone and glycosides accumulate in the seeds [12].

To date, 36 types of aglycones and 17 types of glycosides have been identified mainly in citrus fruits of the family Rutaceae, which is composed of 160 genera and about 2,070 species [13]. The first study of the physiological effects of *Citrus limonoids* reported inhibitory effects on the eating behaviors of armyworms and predatory insects [14]. Strong inhibitory effects on eating were subsequently observed in termites. It has been reported that the ligand activity of the bile acid receptor TGR5 increases the inhibition of tumor formation and the activity of glutathione S-transferase, which is a detoxification enzyme that assists with the excretion of toxic substances by the liver and digestive organs, as well as increasing anti-obesity effects via insulin and increased heat production [15]. The seeds of citrus fruits contain particularly potent limonoids. The metabolic pathway of limonoid biosynthesis in citrus fruits has been nearly elucidated by Hasegawa et al. [16] with the use of ^{14}C -labeled radioisotopes as tracers (**Figure 3**). Within the phloem of the stem, nomilin is synthesized through the metabolism of acetic acid, mevalonic acid,

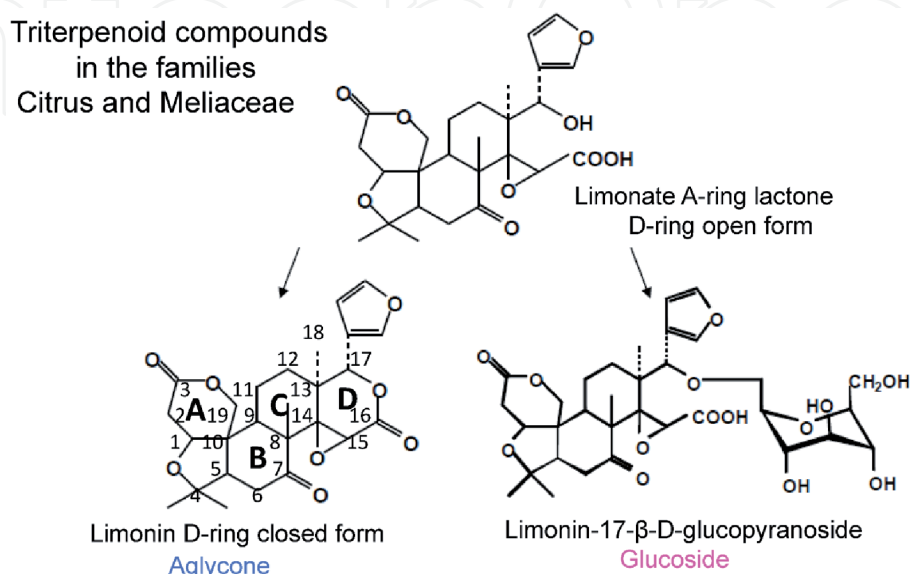


Figure 2.
 D lactone ring structures of limonin.

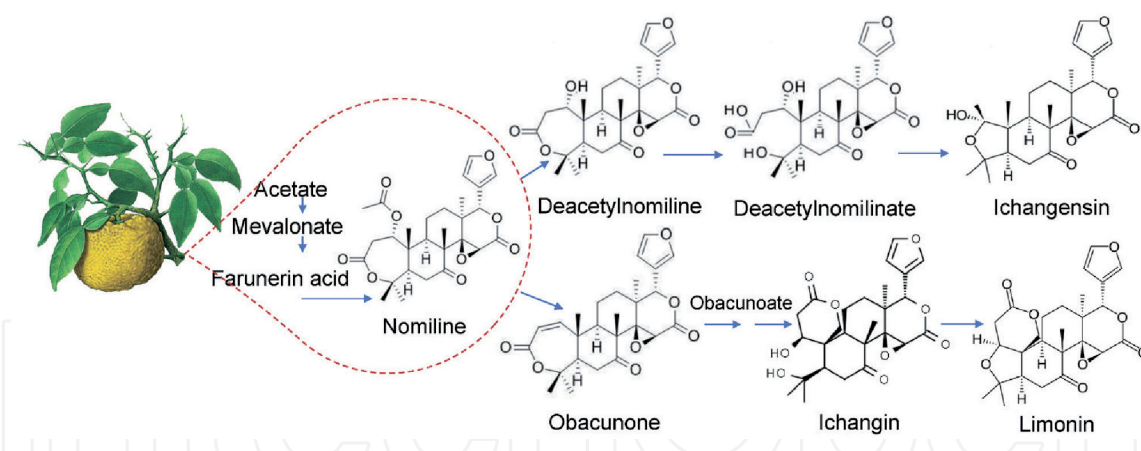


Figure 3.
The limonoid biosynthesis pathway of yuzu seeds.

and furnesole phosphate. Especially in the stems of seedlings, nomilin synthesis becomes very active [17].

Nomilin is synthesized only in the stem and then transferred to the leaves, fruits, and seeds where it is metabolized into other limonoids. Since metabolism proceeds with the D-ring open form, limonoids exist in the D-ring open form in the stems, fruits, and leaves, and mostly form glycosides. In seeds, metabolism to limonoid aglycone with a closed D-ring also proceeds at the same time, so that aglycone and glycoside are accumulated simultaneously. Therefore, the aglycon in the fruit tissue decreases during maturation, but continues to accumulate in the seeds.

2. Bioactive substances of yuzu seeds

Minamisawa et al. [18, 19] has been searching for new antioxidants to maximize the original functions of living organisms with the use of waste resources derived from natural products, including yuzu seeds, that can be regenerated as many times as possible in the human life span. In 2011, the oldest original species of yuzu in Japan was discovered in the village of Mizuo, which is located in the north-west of Kyoto city [2]. This yuzu is characterized by “seedlings” grown from seeds in the land associated with Emperor Seiwa (9th century) and Emperor Hanazono (15th century), and it takes about 20 years to harvest. Most of the citrons cultivated in Japan today originate from the yuzu of Mizuo, which is cultivated mainly by grafting, and the growth is faster than that of seedlings. Since the yuzu of Mizuo is considered to be the finest quality, the fruit is highly demanded by high-end restaurants serving Japanese cuisine in Kyoto. However, yuzu seeds, which are closer to the ancestral citrus, account for 20–30% of the fruit weight, but are discarded as waste after the juice extraction process.

Hence, our team chose to evaluate the this development of active natural resources would encompass the application in nutrition and environmental attributes of yuzu seeds as natural resources with bioactivities [20]. In 2014, we reported the development of a relatively simple technique to simultaneously extract secondary metabolites of yuzu seeds, including expensive limonoids and yuzu seed oil with high total antioxidant capability, from the waste of fully ripe fruits [2]. Yuzu seeds contain higher amounts of fat-soluble limonoid aglycones, water-soluble limonoid glycosides, and oil than other citrus fruits (**Figure 4**).

Analysis of the components of limonoids from yuzu seeds by high-performance liquid chromatography–mass spectrometry identified five limonoid aglycones (deacetylnomilin, limonin, nomilin, obacunone, and ichangensin) and eight

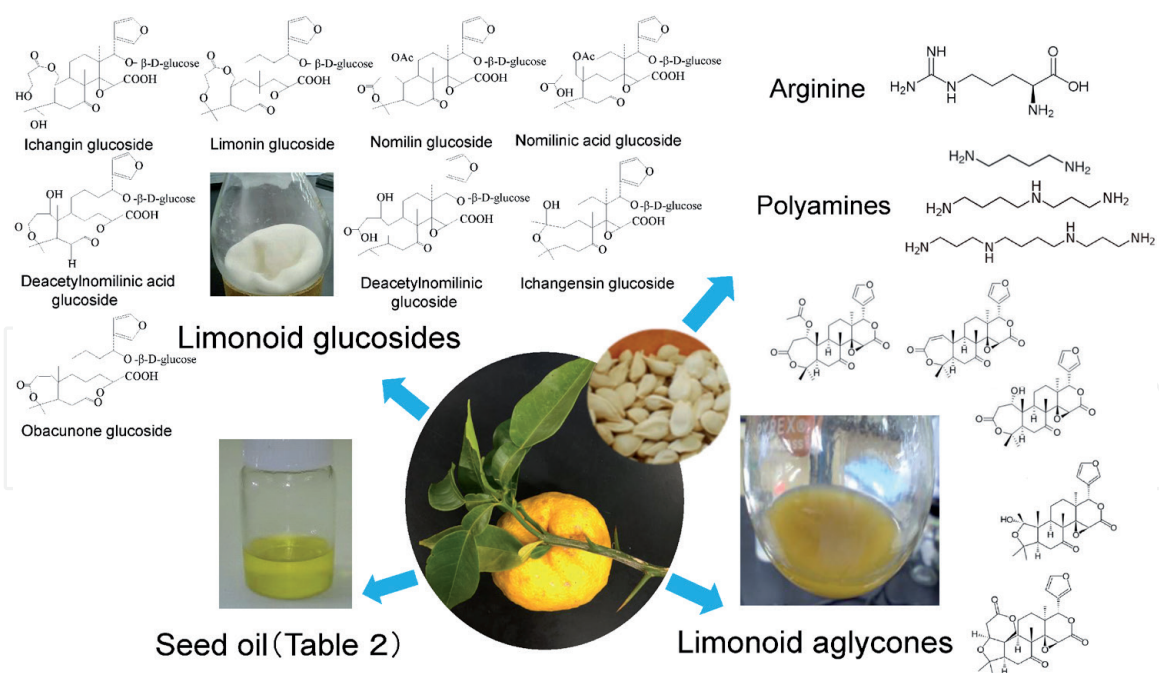


Figure 4.
 The extracted components from Yuzu seed.

limonoid glycosides (limonin glucoside, ichangin glucoside, deacetyl nomilinic acid glucoside, deacetylnomilinic glucoside, nomilin glucoside, nomilinic acid glucoside, ichangensin glucoside, and obacunone glucoside) (**Figure 4**, **Table 1**). Yuzu seed oil extracts (**Table 2**) contain large amounts of oleic and linoleic acids ([2], in preparation). The contents of limonoids extracted from yuzu seeds compared with the results of previous studies are shown in **Table 1** [21–23].

As compared to other citrus seeds, the concentrations of limonoid aglycones extracted from the seeds of yuzu fruit from Kyoto were two- or three-fold greater than in fruits from Tokushima and California (334 vs. 167 and 0.94 mg/g, respectively). According to Nogata [22], the iyokan fruit (*C. iyo*), Valencia orange (*C. sinensis Osbeck*), and hyuganatsu (*C. tamurana Hort. ex Tanaka*) belong to the same family as the daidai (*C. aurantium group V*). Hence, the limonoid compositions of these varieties are similar (**Table 1**). Although the amount of nomilin in the Valencia orange is similar to that in the iyokan and hyuganatsu varieties, the amount of limonin is approximately two-fold greater, while the amount of deacetylnomilinic is higher and that of obacunone is significantly lower.

The yuzu and hanayu (*C. hanaju*) varieties are classified to yuzu group VI. However, both the compositions and amounts of the limonoid aglycones differed markedly between these two species in the present study, which may be attributed to differences in the metabolism of the seeds and fruits [24, 25]. For this reason, the ratio of aglycone to glycosides in mature fruit tissues is mostly due to glycosides, whereas the glycoside content in seeds may be the same or lower than that of aglycones (**Table 1**). These findings indicate that limonoids are biosynthesized completely independently of fruit tissues and seeds.

Nogata et al. [22] pointed out that the high amounts of glycosides in seeds of the iyo and shiikuwasha fruits could be due to the high activity of uridine diphosphate-D-glucose transferase, and perhaps in the yuzu seeds as well. The high limonoid content in the seeds of yuzu fruit grown in Kyoto is thought to be related to the seedling cultivation method. Similar to yuzu seeds, the glycosides deacetyl nomilinic acid glucoside and deacetylnomilinic glucoside, but not ichangensin glucoside, accumulate in hanayu seeds. Ichangensin is reportedly metabolized from nomilin through the intermediaries deacetylnomilinic and deacetyl nomilinic acid [26, 27].

Content (mg/g of dry seeds)								
Seeds	Limonoids aglycones						Total	
	Nomilin	Deacetyl-nomilin	Limonin	Obacu-none	Ichan-gensin	Aglycones	Glucosides	Aglycones/glucosides
Yuzu (<i>Kyoto, Japan</i>)	114	106	95.0	16.7	2.10	334	452	0.74
Yuzu (<i>Tokushima, Japan</i>)	58.4	48.3	54.0	5.20	1.60	167	192	0.87
Yuzu* [21] (<i>California</i>)	0.25	0.22	0.47	—	0.23	0.94		
Hanayu [22]	0.12	0.86	0.54	0.07	—	1.59	15.9	0.10
Shiikuwasha [22]	0.96	—	1.87	0.45	—	3.28	12.7	0.30
Iyo [22]	2.53	0.72	4.57	0.91	—	8.73	4.46	2.00
Hyuganatu [23]	3.73	0.35	4.68	0.28	—	9.04	3.12	2.90
Grapefruit [23]	1.84	1.10	19.1	1.86	—	23.9	6.98	3.40
Lemon [23]	3.03	—	8.95	0.58	—	12.6	6.37	2.00
Valencia [23]	2.30	1.24	10.0	0.08	—	13.6	8.71	1.60
*Units are mg/g of fresh weight.								

Table 1.
Limonoids in various citrus seeds.

Fatty acid	16:0	16:1	18:0	18:1	18:2n-6	18:3n-3	20:0	20:1	24:0
%	19.7	0.5	3.8	37.9	34.7	1.6	0.3	0.2	0.1

Table 2.
The FA content was determined by gas chromatography with the use of a GC-14 gas chromatograph (Shimadzu corporation, Kyoto, Japan) equipped with a DB-1 column (30 m, 0.25 mm, Agilent Technologies, Inc., Santa Clara, CA, USA), which was maintained at a constant temperature of 300°C. The yield of yuzu seed oil was 100 mg/g of dry seeds.

The hanayu are, therefore, different from other citrus varieties. Although both belong to the same yuzu group, there are differences in characteristics, such as aglycones contents.

Several *in vitro* studies have shown that limonoid components mediate the antioxidant properties of citrus. Reactive oxygen is believed to be a factor in diseases with underlying cellular disorders [28]. Modern lifestyles and diets involve a number of factors that can increase the production of active oxygen species, which can overwhelm the body’s self-regulating defense mechanisms [29, 30]. One way to protect oneself from active oxygen species is to consume foods containing antioxidants. One of the main reasons for our interest in antioxidants is the link between active oxygen species and aging.

Limonoids components of *C. junos* are known to possess a vast range of biological activities, including antioxidant functions, protective effects on vascular endothelial cells [31], and anti-carcinogenic activities [15, 20, 32–34].

Study also evaluated the *in vitro* antioxidant activities of yuzu seed aglycones, glycosides, and oil extracts. Notably, the yuzu seed oil, the potential extracts had high antioxidant activities due to the presence of lipophilic aglycones (Figure 5, new unpublished data). Yuzu seed oil is a semi-drying oil that contains large amounts of unsaturated fatty acids (FAs), mainly oleic acid and linoleic acid, in addition to a lot of palmitic acid.

Virgin yuzu seed oil, which is obtained by a pressing process without heating, contains about 2% of limonoid aglycones. Pure oil with the composition shown in Table 2 can be obtained by heating and drying roasted yuzu seeds, followed by extraction with an organic solvent, such as hexane, and purification.

Lipophilic limonoid aglycones, which were extracted from the residual extracts of yuzu seed oil [2], were composed of the following concentrations of limonoids per gram of dry seeds: deacetylnomilin, 105 mg; limonin, 95 mg; nomilin, 115 mg; obacunone, 17 mg; and ichangensin, 2.1 mg.

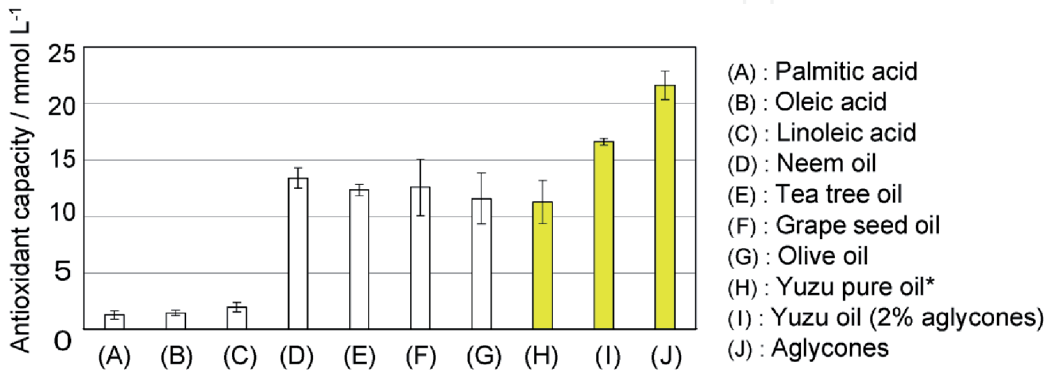


Figure 5.
Total potential antioxidant activities of various plant seed oils and yuzu aglycones by the total potency of antioxidants that are soluble in oil method [35]. Measurements were performed 4–6 times or more. The inhibition ratio is presented as the average value ± standard deviation (S.D.).

The total potential antioxidant capacity of yuzu seed oil and lipophilic limonoid aglycones was measured by utilizing the reduction reaction of copper ($\text{Cu}^{++}/\text{Cu}^{+}$) [35]. Many other plant oils, including olive oil [36], tea tree oil, grape seed oil [37], and neem seed oil [38], which have strong antioxidant activities, were measured at the same time for comparisons. Among all of the tested plant seed oils, limonoid aglycones extracted from yuzu seeds had the highest antioxidant capacity, followed by yuzu seed oil, neem seed, grape seed, tea tree, olive oil, and pure yuzu seed oil. The antioxidant capacity of pure yuzu seed oil was approximately 6–9-fold greater than that of palmitic acid, oleic acid, and linoleic acid.

While water-soluble antioxidants are rapidly excreted through the urine if an excessive amount is ingested, fat-soluble antioxidants are adsorbed onto lipoproteins and cell membrane lipids, and are therefore considered to exhibit a higher activity in the body. For this reason, fat-soluble antioxidants are expected to be beneficial in preventing diseases caused by oxidative stress. Vitamin E, oryzanol, and carotenoids are well-known examples of fat-soluble antioxidants. Neem (*Azadirachta indica*) seed oil, which has the same total antioxidant capacity as yuzu seed oil, contains the triterpene derivative azadirachtin, which is similar to the triterpene limonoids of yuzu seed oil, which is a potent insect repellent [39]. Press-extracted virgin olive oil contains oleocanthal that has a potent anti-inflammatory effect strikingly similar to that of ibuprofen. Both of these molecules inhibit the same cyclooxygenase enzymes in the prostaglandin-biosynthesis pathway [40].

The result in **Figure 5** suggest the presence of other types of fat-soluble antioxidants. Limonoid aglycones also contribute to the high antioxidant capacity of yuzu seed oil.

3. Yuzu seeds contain arginine and polyamines (PAs)

Atherosclerosis has become a serious health concern worldwide, as one-third of the global population is at risk for diseases associated with arteriosclerosis, which accounts for about half of deaths in developed countries. In particular, cardiovascular disease (CVD), which is a consequence of atherosclerosis, is the leading cause of death in industrialized nations. Besides lifestyle habits, body weight, socio-economic factors, and certain pre-existing conditions, a number of foods seem to play a role in the incidence of CVD [41, 42]. In addition, many studies have suggested the importance of inflammation in atherosclerosis and CVD [43, 44].

Some food components with anti-inflammatory properties can decrease the risk of CVD [44, 45]. Many foods contain wide-ranging concentrations of natural PAs, such as spermidine (Spd) and spermine (Spm), which suppress the synthesis of pro-inflammatory cytokines [46, 47]. In particular, an epidemiological survey of Westerners found that “people who eat cheese or yogurt every day are less likely to have myocardial infarction.” [48]. The Japanese consume a lot of traditional fermented foods, mainly soybeans, which are thought to suppress arteriosclerosis [49]. PAs concentrations are relatively high in yogurt, cheese, and traditional Japanese foods. PAs are aliphatic amines that are essential for the growth of all living cells [50]. PAs exist primarily in association with RNA and are involved in promoting the synthesis of specific proteins and overall protein synthesis via the ribosome activation. As shown in **Figure 6**, the PAs comprising Put ($\text{NH}_2(\text{CH}_2)_4\text{NH}_2$) \rightarrow Spd ($\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$) \rightarrow Spm ($\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$) are produced from arginine via ornithine or agmatine [51].

PAs have been implicated in the regulation of several growth and development processes in plants, including cell division, morphogenesis, flower initiation, pollen

chromatography, as well as the arginine and free arginine contents, as determined by automated amino acid analysis, of various citrus fruits are shown in **Table 3**.

As compared with the juice and peel of yuzu and lemons, the seeds contain higher quantities of PAs and arginine. The Put contents are high in all citrus fruits, but the quantities of Spd and Spm in yuzu seeds were 5–23-fold greater than the reference values. The PA contents of yuzu and lemon fruit are not high as compared to legumes [57], but when the limonoid and arginine contents are also considered, these fruits have high levels of functional constituents.

As mentioned earlier, limonoids and PAs have various bioactivities and reportedly have strong anti-inflammatory capabilities. Hence, the potential antioxidant activities (i.e., H_2O_2 -scavenging activity, 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity, and inhibition of superoxide dismutase [SOD] and antioxidants with SOD-like activities) of PAs (Put, Spd, and Spm) and arginine were investigated (in preparation). The results showed that these compounds have no antioxidant activities or only weak (less than 10%) inhibitory potential. As reported in many studies, the anti-inflammatory activities of PAs and arginine are due to factors other than antioxidant capacity.

4. Yuzu seed limonoids or Spm increased survival of mice with Sandhoff disease

In our previous study, we investigated the life-extending effect of limonoids (lipophilic limonoid aglycones) and Spm as an exogenous anti-inflammatory component in a mouse model of Sandhoff disease (SD), which is a lysosomal disease [58]. Lysosomal storage disorders are caused by functional defects of proteins that are essential for normal lysosome function, such as enzymes that play critical roles in the intracellular digestion of glycoproteins, glycolipids, glycosaminoglycans, and other macromolecules [59]. SD is an autosomal recessive hereditary disease [60]. The gangliosides GM2 and GA2 accumulate in the nervous system, resulting in severe developmental and neurological disorders, and death, which usually occurs during infancy because of the lack of effective treatment methods. Neurological dysfunction is the major clinical manifestation of GM2 gangliosidosis [61–64].

SD mice present with trembling, startled responses, and decreased motor activities from 11 to 15 weeks of age (105 days) due to damage caused by microglial activation, macrophage infiltration, and oxidation associated with the accumulation of glycolipids. It has been suggested that inflammation may be fatal [51]. The therapeutic effects of enzyme replacement therapy and anti-inflammatory drugs have been reported [65]. Inflammation due to the accumulation of lipids is inhibited by antioxidant and anti-inflammatory treatments, which can delay disease progression, but no cure exists at present.

We consider the degeneration of the nervous system might be rooted in oxidative stress and inflammation. Given that dietary interventions can moderate these phenomena, consuming foods with antioxidants and anti-inflammatory components, such as limonoid aglycons (limonoids) and Spm, could effectively combat or minimize neurological damage. Therefore, the inhibition of SD pathologies could be promoted by factors other than suppressing the storage of gangliosides.

Preventing inflammation appears to be one of the most effective approaches for increasing longevity [66, 67]. To test this hypothesis, the life spans of SD mice treated with limonoids or Spm were assessed. The prognostic outcomes of SD mice, a typical model of abnormal glycolipid metabolism in humans, were observed after administration of limonoids extracted from yuzu seeds and Spm. The treated mice

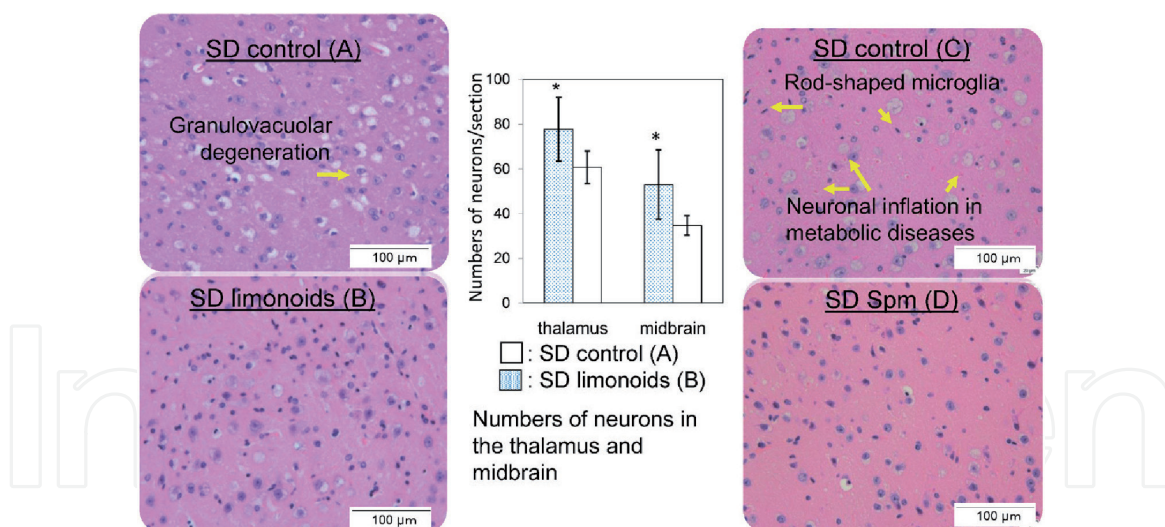


Figure 7. H&E-stained thalamus sections of control SD mice (A and C) and SD mice administered yuzu limonoids (B) and Spm (D). Enlarged cells with ganglioside storage are indicated. The numbers of neurons in the thalamus and midbrain of SD mice administered limonoids (B) and control SD mice (A). Data are presented as the mean \pm S.D. * $p \leq 0.05$ (Student's *t*-test).

lived significantly longer than untreated littermates (9–10%, $p < 0.01$) and had a slower rate of disease progression ($p < 0.01$) [58]. When limonoid treatment was combined with Spm therapy, synergy resulted in a maximum improvement of 12% in survival ($p < 0.001$) (in preparation). The hematoxylin and eosin (H&E) staining results of thalamus sections of SD mice following administration of limonoids or Spm are shown in **Figure 7**.

H&E staining results of the neural tissues of the SD control mice (A) and (C) correspond to SD mice treated with limonoids (B) and Spm (D), respectively.

Gangliosidosis and inflammatory/autoimmune diseases are characterized by degeneration and the accumulation of fat, granulovacuolar degeneration, rod-shaped microglia, and neuronal inflammation in metabolic diseases, as determined by analyses of pathological tissues (Tokyo Metropolitan Institute for Medical Science). The characteristic degeneration was clearly decreased in SD mice treated with limonoids or Spm. The numbers of neurons in the thalamus and midbrains of SD mice treated with limonoids were higher than those in the control SD mice. These results demonstrate that inflammation contributes to disease progression and the anti-inflammatory effects of Spm and limonoid therapies as a potential adjunctive approach to slow the clinical course of inflammatory diseases.

5. Bacterial flora analysis of SD mouse feces by the 16S ribosomal DNA (16S rDNA) terminal restriction fragment length polymorphism (T-RFLP) method

PAs possess anti-inflammatory activities by inhibiting the synthesis of inflammatory cytokines by macrophages and the regulation of nuclear factor- κ B activation, which are closely associated with maintaining the intestinal mucosal barrier function [68]. Bilateral signals between the intestine and brain are involved in the control of nerve, hormone, and immune activities, as well as prolonging longevity [69]. Recent studies have shown that bilateral signals between the brain and intestine are important for maintaining homeostasis and extending the life span [70]. In particular, the functions mediated by PAs may be involved in metabolism by indigenous intestinal bacteria and the health of the host [71].

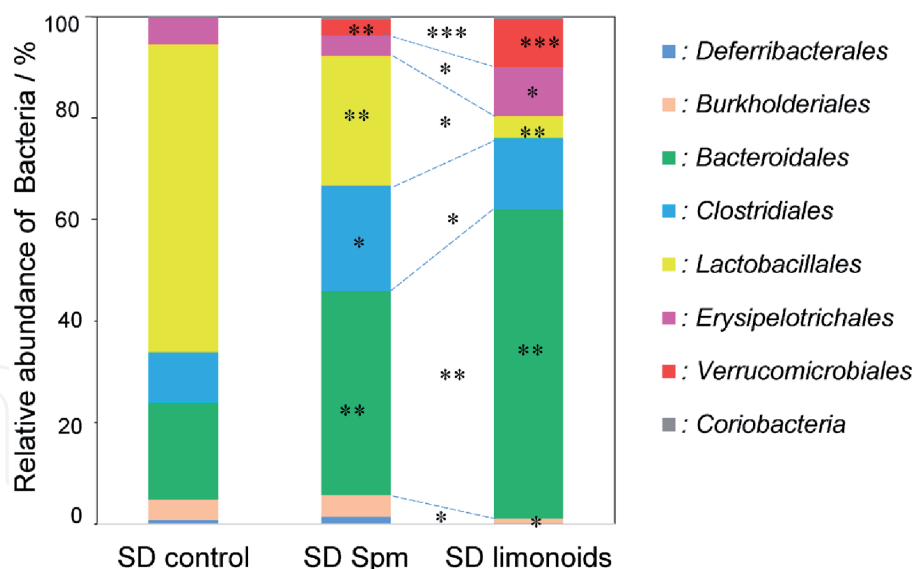


Figure 8.

Estimated ratios (%) of the taxonomic categories of the bacterial flora at the order level were identified by T-RFLP analysis of 16S rRNA in the feces of SD mice at 12 weeks of age in the control, Spm, and limonoids groups ($n = 9/\text{group}$). Values are presented as the mean \pm S.D. data of the treatment groups are plotted against those of the control group. * $p \leq 0.05$, ** $p \leq 0.001$, and *** $p \leq 0.0001$ vs. the untreated control SD mice or each (dashed line----). All experiments were performed at least three times.

At 12 weeks of age for which the survival period was extended by limonoids or Spm, T-RFLP analysis of 16S rRNA was performed to classify the intestinal microbiota at the order level for each mouse group (**Figure 8**). The results showed that the taxonomic groups of the bacterial flora in feces after administration of limonoids or Spm were completely different from those of the SD control mice.

The bacterial flora in feces after administration of limonoids or Spm had increased proportions of *Bacteroidales* and *Clostridiales*. However, *Lactobacillus* was remarkably prevalent in feces of the SD control mice. The abundance of *Clostridiales* were significantly increased in the feces of SD mice treated with Spm, whereas *Bacteroidales* were increased in the feces of SD mice treated with limonoids. The administration of Spm or limonoids slightly increased the proportion of *Erysipelotrichales*.

It is generally known that the abundance of *Erysipelotrichaceae* is increased due to fat accumulation in mice [72]. In this case, it was possible that the bacterial flora of the SD control mice caused dysbiosis [73]. In the SD control mice, dysbiosis may have been due to suppressed absorption of dietary fats and other nutrients. Even more interesting was the significant appearance of *Verrucomicrobiaceae* in feces after the administration of limonoids or Spm, which were not found in feces of the control SD mice. *Verrucomicrobiaceae* include mucin-degrading bacteria that are also present in the human intestine, and especially *Akkermansia*, which promote the suppression of obesity, diabetes, and inflammation [74, 75].

It will be necessary to investigate the specific bacteria involved in more detail. Unfortunately, the T-RFLP method made it difficult to analyze the bacterial flora in more detail, and it was not possible to identify particular species. We are currently preparing a report of the findings of next-generation sequencing that allowed for more detailed classification.

6. Short-chain fatty acid (SCFA) production in SD mouse feces

There have been many reports of the relationships between chronic inflammatory diseases and the intestinal bacterial flora that have helped to clarify the balance

between the intestinal ecosystem and diseases related to the intestinal tract. For example, genetic abnormalities and the breakdown of the intestinal ecosystem have been detected in inflammatory bowel disease [76]. In particular, members of the genus *Clostridium* promote the production of butyric acid, induce an immune response in the intestinal mucosa, and promote the differentiation of regulatory T cells (Tregs) that contribute to suppression. Thus, changes in intestinal *Clostridium* are considered to be closely related to the onset of inflammatory bowel disease [77, 78]. It has been reported that the SCFAs produced by intestinal bacteria may function as bio-modifying factors. Hence, the SCFA composition of feces from the same mice at 12 weeks were determined (Figure 9).

The production levels of SCFAs comprising acetic acid, propionic acid, and butyric acid were increased in mice administered limonoids or Spm as compared to SD control mice. In particular, the production levels of all SCFAs were higher in SD mice following administration of limonoids or Spm. The experimental results demonstrated differences between the fecal microflora composition and these metabolites after administration of limonoids or Spm. Butyric acid is a SCFA that is produced by clostridia [78].

As shown by the results presented in Figures 8 and 9, the addition of Spm to the diet clearly increased the proportion of *Clostridiales* and butyric acid in feces. Previous metabolomic analyses have shown that butyric acid contributes to the induction of Treg differentiation in the colonic mucosa. Thus, butyric acid functions as a histone deacetylase inhibitor and as an immunomodulator responsible for inducing Treg differentiation in the colonic mucosa, as well as the activation of dendritic cells [79]. Acetic acid produced by intestinal bacteria suppressed colitis in a mouse model by promoting apoptosis via the GPR43 receptor expressed by neutrophils and plays a central role in the inflammatory reaction [80, 81]. Furthermore, the addition of limonoids seems to contribute to the production of acetic acid and propionic acid as well as butyric acid.

Acetate, butyrate, and propionate are produced by members of the intestinal microbial community through fermentation of dietary fibers and starches, which are unable to be broken down by host metabolism [82]. In turn, these metabolites are sensed by host cells through various G-protein coupled receptors, known as free

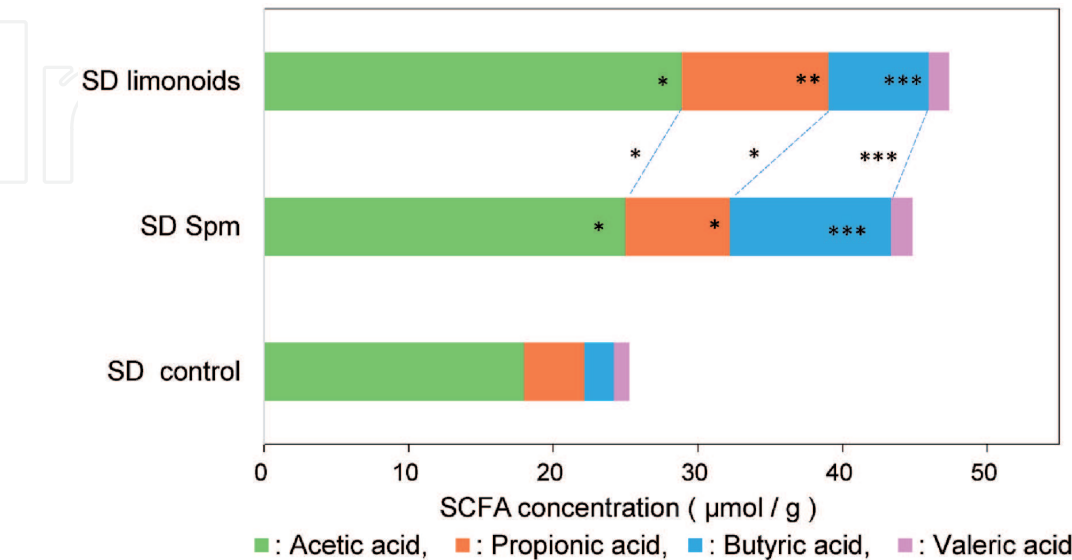


Figure 9. SCFA contents (μmol/g) in feces of SD mice in the control, Spm, and limonoid groups. SD mice were treated at 12 weeks of age (n = 9/group). Values are presented as the mean ± S.D. data of the treatment groups are plotted against those of the control group. *p ≤ 0.05, **p ≤ 0.001, and ***p ≤ 0.0001 vs. the untreated control SD mice or each (dashed line----). All experiments were performed at least three times.

fatty acid receptors, and intracellular peroxisome proliferator-activated receptor gamma. Furthermore, SCFAs can also regulate cellular responses through inhibition of histone deacetylases. Examples of the effects of SCFAs on the host include differentiation of Tregs and macrophages, and downregulation of pro-inflammatory mediators. These effects underline the fine balance that SCFAs help to maintain between intestinal immunity and inflammation [82–84].

These results suggest that yuzu conveys anti-inflammatory and lipid metabolism-promoting activities in mice following administration of limonoid aglycones and Spm. Thus, the metabolites of intestinal bacteria may be indirectly involved in suppressing the inflammatory mechanism to directly enhance the health of the host. Furthermore, administration of limonoids or Spm improved the proportions of beneficial bacteria in the intestinal flora and associated metabolites. In the healthy intestinal tract, the microbiota and gut-associated immune system are assumed to be at a dynamic homeostatic equilibrium [85], but the inflammation process may undermine this balance. We consider that the human lifespan can be extended by inhibiting inflammation via control of the intestinal microbiota.

However, it was not possible to elucidate the mechanisms underlying the effects of limonoid aglycones and Spm on the extended life span of SD mice. Thus, in order to clarify the anti-inflammatory effects of yuzu seed extract, limonoids, and Spm, as well as to widely apply yuzu to promote health and enhance longevity, it will be necessary to determine the composition of the bacterial flora based on detailed metagenomic analyses of 16S rRNA. Furthermore, it will be necessary to analyze the anti-inflammatory effects of limonoids and Spm in yuzu seed extracts at the gene level.

PAs quantities have reference values. The reference values for the PAs contents of legumes are also shown in **Table 3**. The values for Japanese produced yuzu and lemons are shown, as well as the reference values for other citrus fruits. No previous studies reported the quantities of arginine in citrus fruits, so only the compared PA quantities are indicated by reference values. The reference values for the PA contents of legumes are also shown.

7. Conclusions

Yuzu is a natural and renewable resource of limonoids, arginine, and PAs. The results of the present study suggest that yuzu limonoids and Spm improved the proportions of beneficial bacteria and their metabolites in the intestinal flora. Thus, the ingestion of fruits that contain high concentrations of specific ingredients may be a simple method to suppress inflammation, thereby enhancing immune function, improving intestinal health, and increasing lifespan. In other words, our this study demonstrated the possibility that bilateral signals between the brain and intestine are important for maintaining homeostasis and extending lifespan. However, it was not possible to examine the physiological effects of limonoids and Spm. Thus, future studies are needed to evaluate the effects of limonoids and Spm on metabolism and the immune response, and to explore the potential of these molecules as natural antioxidants/antibiotics for lysosomal diseases, such as SD.

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Conflict of interest

The authors declare no conflict of interest.

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