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Alcoholic fermentation with Pichia kluyveri could improve the melatonin bioavailability of orange juice

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ABSTRACT

Fermentation of orange juice (OJ) by Pichia kluyveri enhances the content of melatonin, a molecule with potent antioxidant effect. This study explores the levels of urine 6- sulfatoxymelatonin (6-SMT) in healthy subjects after fermented orange juice (FOJ) intake, and their association with antioxidant activity status. Nine participants ingested 500 mL of FOJ and their urine was collected at baseline and after 2, 5, 10, 15 and 24 h. After a two-week washout period, the intervention was repeated with OJ. 6-SMT levels were quantified by ELISA and antioxidant activity by TAC, FRAP and ORAC assays. A significant increase in both 6-SMT levels and antioxidant activity in urine was observed after FOJ ingestion compared to OJ. A positive correlation between TAC and 6-SMT levels was observed only after FOJ intake. This study shows for the first time that fermentation process increases melatonin bioavailability of OJ associated with an enhancement in antioxidant status.

1. Introduction

Persistent oxidative stress in the organism has been associated with various diseases, such as neurodegenerative diseases (Alzheimer's and Parkinsońs disease), degenerative retinopathy (glaucoma and macular degeneration), cardiovascular diseases, brain ischemia/reperfusion injury (cardiac attack and stroke), and cancer, among others (Dubois-Deruy, Peugnet, Turkieh, & Pinet, 2020; Forman & Zhang, 2021; Sharifi-Rad et al., 2020).

Melatonin (*N*-acetyl-5-methoxy tryptamine, MLT) is a ubiquitous molecule derived from the amino acid tryptophan, which is involved in numerous biological functions, such as antioxidant and anti-inflammatory activities and regulation of circadian rhythms, seasonal breeding, glucose metabolism, and thermogenesis (Asghari, Abdollahi, de Oliveira, & Nabavi, 2017; Manchester et al., 2015; Motilva, García-Mauriño, Talero, & Illanes, 2011; Pahlavani et al., 2019; Sun et al., 2018; Tengattini et al., 2008). In addition, recent studies have shown the involvement of MLT in protecting against chronic diseases such as diabetes, obesity, gastrointestinal and immune disorders, cardiovascular and neurological diseases, and cancer (Favero et al., 2017; Fernández Vázquez, Reiter, & Agil, 2018; Gunata, Parlakpinar, & Acet, 2020; Ma et al., 2019; Moradkhani et al., 2020; Sun, Gusdon, & Qu, 2016).

MLT is a well-known potent scavenger of reactive oxygen species (ROS), such as the hydroxyl radical, peroxyl radical, superoxide anion radical, and hydrogen peroxide (Favero et al., 2017). Not only MLT, but also several of its metabolites, can detoxify free radicals and their

Abbreviations: MEL, melatonin; 6-SMT, 6- sulfatoxymelatonin; OJ, orange juice; FOJ, fermented orange juice; TAC, total antioxidant capacity; ORAC, Oxygen Radical Absorbance Capacity; FRAP, Ferric Reducing Antioxidant Power; TMB, 3,3́,5,5́-tetramethylbenzidine.

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derivatives (Jeon et al., 2016; Kalogiannis, Delikatny, & Jeitner, 2016). The process by which MLT and its metabolites successively scavenge ROS and reactive nitrogen species (RNS) is called the free radical scavenging cascade. This cascade reaction makes MLT highly effective and explains how it has added value over other conventional antioxidants (Tan, Manchester, Terron, Flores, & Reiter, 2007). In this line, aging is associated with a significant reduction in endogenous MLT secretion (Karasek & Reiter, 2002), which promotes the increase of oxidative stress and other metabolic changes (M Hill et al., 2013).

MLT has also been identified in a very large number of plant species, in plant food and medical herbs. Therefore, the consumption of products rich in MLT (wine, beer, walnuts, fermented orange juice and other food or beverage) can increase plasma levels of MLT or 6-sulfatoxymelatonin (6-SMT), the main excreted urinary metabolite of MLT (Fernández-Pachón et al., 2014; Garrido et al., 2010; Maldonado, Moreno, & Calvo, 2009; Nduhirabandi, Lamont, Albertyn, Opie, & Lecour, 2016; Oba, Nakamura, Sahashi, Hattori, & Nagata, 2008; Reiter, Manchester, & Tan, 2005; Sae-Teaw, Johns, Johns, & Subongkot, 2013). The potential antioxidant activity of 6-SMT has been showed through association between 6-SMT levels and urine antioxidant activity (Ortiz et al., 2011; Bravo et al., 2013; Bejarano et al., 2014).

Exogenously administered, MLT is quickly distributed throughout the organism, crosses all morphophysiological barriers, and enters easily into cells (Favero et al., 2017). However, MLT has a very short average life in the blood (a range of 20 to 40 min) before its metabolism and elimination occur, so a large amount of MLT in the blood cannot persist for more than many hours (Härtter, Grözinger, Weigmann, Röschke, & Hiemke, 2000). In addition, urinary 6-SMT levels are widely considered to be a good indicator of MLT in plasma, showing a correlation with plasmatic levels (Johns, Johns, Porasuphatana, Plaimee, & Sae-Teaw, 2013). Thus, urinary 6-SMT levels can be used to evaluate the bioavailability of MLT from foods and beverages.

Orange juice (OJ), the most consumed fruit juice worldwide, contains a wide range of micronutrients and phytochemicals that have been attributed preventive effects against the onset of several non-communicable diseases, such as cardiometabolic diseases, neurological disorders, and some types of cancer (Kean et al., 2015; Maugeri et al., 2019; Rech Franke, Guecheva, Henriques, & Prá, 2013; Rees, Dodd, & Spencer, 2018). The list of bioactive compounds of OJ includes vitamin C, (poly)phenols (mainly flavanones), carotenoids (xanthophylls, cryptoxanthins, carotenes), folate, and MLT (Escudero-López et al., 2013). We have previously shown that alcoholic fermentation improves the content of bioactive antioxidant compounds (Escudero-López et al., 2013), including MLT (Fernández-Pachón et al., 2014) and its precursor tryptopahn (Cerrillo et al., 2015), with respect to OJ. Previous studies have also reported increased serum MLT concentrations after tryptophan ingestion as a result of gastrointestinal MLT synthesis (Sánchez, Sánchez, Paredes, Rodriguez, & Barriga, 2008). Thus, fermented orange juice (FOJ) could be a good source of both MLT and its precursor tryptophan, leading to endogenous biosynthesis of MLT.

Although the bioavailability of OJ and FOJ polyphenols has been investigated (Pereira-Caro et al., 2014), currently no information is available on oral bioavailability of MLT after consumption of OJ versus FOJ. In addition, no bioactive effects of MLT-containing FOJ have been previously reported. Since the influence of alcoholic fermentation on the bioavailability of MLT has not previously been studied in any fruit or juice derived, the objective of the present study was to conduct a pilot study aiming to determine the levels 6-SMT in urine of nine healthy subjects after intake of OJ versus FOJ, as well as to determine the antioxidant capacity of urine of the subjects.

2. Materials & methods

2.1. Subjects

The study included nine healthy subjects (six women and three men) aged 21–25 years with a mean (±SD) body mass index (BMI) of 20.6 ± 1.8 kg/m^2. Eligibility was based on routine hematological and biochemical laboratory tests, medical history, anthropometric measurements, and a health and lifestyle questionnaire. Exclusion criteria were: 1) the presence of a chronic disease (cardiovascular disease, diabetes, cancer, chronic obstructive pulmonary disease, metabolic syndrome), overweight/obesity, and kidney or liver failure; 2) screening blood tests with non-standard values (out to the laboratorýs reference ranges of haematological and biochemical markers); 3) the intake of any medication or nutritional supplement containing MLT or other bioactive compound in the previous 4 weeks; 4) any smoking habit; and/or 5) alcohol consumption of > 2 drinks/day.

All participants gave written informed consent to participate in the study, which followed the principles of the Declaration of Helsinki and was approved by the Clinical Research Ethics Committees of Virgen del Rocío Hospital and Pablo de Olavide University, Seville, Spain (IEC 2013PI/022).

2.2. Production and composition of fermented orange juice

Grupo Hespérides Biotech S.L. (Seville, Spain) and Mitra Sol Technologies S.L. (Alicante, Spain) produced FOJ using a commercial pasteurized OJ from Citrus sinensis (L.) var. Navel late. Controlled alcoholic fermentation of OJ was carried out for 10 days at 22 °C in 100 L stainless steel tanks (semi-industrial scale) under aseptic conditions using Pichia kluyveri var. kluyveri (previously isolated from the natural microbiota of orange fruit), which ferments reducing sugars. The alcohol content of the resulting product is low (1 % v/v). The FOJ was pasteurized (25 L/h) at 80 °C for 30 s in a semi-industrial tubular pasteurizer (Mipaser Prototype, Murcia, Spain) and then cooled to 10 °C in an ice-water bath. The juice was then carbonated to a pressure of 0.44 × 10^5 Pa and aseptically poured into aluminum containers (250 mL), which were stored at 4 °C until their consumption. The quality parameters of OJ and FOJ were obtained using the methodology described by The International Organisation of Vine and Wine (OIV, 2017). Tryptophan, total (poly)phenols, and ORAC values were quantified according to previous studies (Cerrillo et al., 2015; Escudero-López et al., 2016; Castello et al., 2020).

2.3. Experimental design

The study was a controlled, blinded, and crossover intervention performed at Pablo de Olavide University (Seville, Spain). Volunteers received a list of MLT-rich foods to avoid for 48 h before the study and throughout its duration. The subjects arrived at the Pablo de Olavide University after fasting overnight for 12 h. Following a baseline urine collection at 8:30 h (0 h), 500 mL of FOJ was consumed under supervision in 15 min. The subsequent accumulated urine samples were then collected throughout the following 24 h at times 2 h (10:30 h), 5 h (13:30 h), 10 h (18:30 h), 15 h (23:30 h), and 24 h (8:30 h of the next morning) after the beverage intake. Urine samples were collected in the BD vacutainer® urine collection tube (BD, Franklin Lakes, NJ, USA), aliquoted in the centrifuge tube, and stored at − 80 °C until the analysis. After urine collection at 13:30 h, the subjects consumed a standardized lunch low in MLT [sandwich with two slices of bread (60 g), cooked turkey (40 g), and a slice of low-fat cheese (20 g) plus non-fat yogurt (125 g)], which provided 245 kcal from 3.2 g total fat, 20 g total protein, and 34.2 g total carbohydrates. After urine collection at 18:30 h, subjects consumed a similar dinner. No other food or beverage was allowed, except water (ad libitum), during the urine sampling period. After a two-week washout period, the intervention was repeated but with the consumption of 500 mL of OJ instead of FOJ. All participants completed the study, and no adverse effects were reported during the clinical trial.

2.4. Melatonin quantification

The MLT content of OJ and FOJ was measured using the MLT ELISA kit (Enzo, NY, USA) following the manufacturer's instructions. A calibration curve of MLT (0–50 ng/mL) was used to obtain the results. 6-SMT levels were assayed in the urine samples using the Melatonin-Sulfate Urine ELISA (IBL International GMBH, Hamburg, Germany) following the manufacturer's instructions. Briefly, urine samples diluted 1:50 were incubated with 6-SMT conjugated with peroxidase and an anti-6-SMT antibody for 2 h. After washing, 3,3́,5,5́-tetramethylbenzidine (TMB) substrate was added, and absorbance was measured at 450 nm (reference wavelength: 620 nm) in a Multiskan FC reader (Thermo Scientific). A calibration curve of 6-SMT (0–420 ng/mL) was used to obtain the results. The standard curves of both MLT and 6-SMT kits are presented in the Figure S1 of the supplementary material. The levels of 6-SMT were corrected to those of creatinine for each sample.

Creatinine was measured in the same samples by a kinetic color test (Jaffé method) on a Beckman Coulter AU2700 analyzer (Beckman Coulter, Inc., Brea CA) using manufacturer's reagents. Creatinine forms a yellow-orange compound with picric acid in an alkaline medium. The rate of change in absorbance at 520/800 nm is proportional to the creatinine concentration in the sample. The test is linear within a concentration range of 88–35,360 μmol/L (1–400 mg/dL) for urine. The lowest detectable level was established using urine settings on an AU2700 analyzer was established at 0.1 μmol/L (0.001 mg/dL) and an intra-and inter-assay CV of 0.96 % and 2.48 %, respectively. The Beckman Coulter AU2700 analyzer is calibrated daily.

2.5. Antioxidant activity

Urine antioxidant activity was evaluated by TAC (Total Antioxidant Capacity), FRAP (Ferric Reducing Antioxidant Power), and ORAC (Oxygen Radical Absorbance Capacity) assays. The ORAC assay was performed according to Ou et al. (Ou, Hampsch-Woodill, & Prior, 2001), diluting urine samples (1:300) in phosphate buffer (75 mM, pH 7.4). Fluorescence was measured (λ_{ex} 460 nm, λ_{em} 515 nm) every 5 min for 120 min. The area under the curve was calculated. The results were obtained from a standard curve using different concentrations of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (2–38 μM). The FRAP assay followed the method of Delgado-Andrade et al. (Delgado-Andrade, Rufián-Henares, & Morales, 2005), diluting urine samples (1:20) in distilled water. The absorbance was recorded at 595 nm. The results were obtained from a calibration curve using different concentrations of Trolox (100–1600 μM). The TAC was determined by colorimetric assay according to the manufacturer's instructions (Cell Biolabs, San Diego, CA, USA). The absorbance was recorded at 490 nm. A calibration curve using different uric acid concentrations (0.039–1 mM) was used to calculate the results. Antioxidant activity measurements were recorded on a Synergy™ HT-multimode microplate reader (Biotek Instruments). The representative standard curves are showed in the Figure S2 of the supplementary material.

2.6. Statistical analysis

Data were analyzed with GraphPad Prism v8 software (San Diego, CA, USA). An ANOVA model with repeated measures + Bonferroni post-hoc test was performed to compare the values of 6-SMT/creatinine, TAC/creatinine, FRAP/creatinine, and ORAC/creatinine along the time-points. Additionally, the U-Mann Whitney test has been used to compare both groups (OJ vs FOJ) at each time-point. To analyze the area under the curve (AUC) values, the nonparametric U-Mann Whitney test was applied. Values of $p \leq 0.05$ were considered statistically significant.

3. Results

3.1. Fermented orange juice composition

The quality parameters, bioactive compounds and antioxidant activity of OJ and FOJ are shown in Table 1. We observed significantly lower values of pulp, ascorbic acid, total sugars, and reducing sugars in FOJ compared to OJ ($p < 0.05$). No significant changes in MLT content between OJ and FOJ were observed. However, the tryptophan level was higher in FOJ compared to OJ ($p < 0.05$). Total (poly)phenols content was also measured and the value reached in FOJ was higher than that of OJ ($p < 0.05$). The antioxidant activity (ORAC value) was similar in both beverages.

3.2. Effect of FOJ ingestion on 6-SMT levels

Fig. 1 shows the concentration of 6-SMT/creatinine in urine samples throughout the experiment (0 – 24 h) after consumption of OJ or FOJ (Fig. 1A) and the AUC of 6-SMT/creatinine levels (Fig. 1B). A non-significant increase in 6-SMT was observed after 5 h of FOJ ingestion (128.18 ± 80.38 ng/mg) compared to 0 h (68.93 ± 38.87 ng/mg, $p = 0.008$) (Fig. 1A). Furthermore, this value reached after 5 h of FOJ intake was significantly higher than that observed after 5 h of OJ consumption (46.86 ± 29.10 ng/mg, $p = 0.015$).

To evaluate the total levels of 6-SMT after FOJ or OJ ingestion, the AUC was calculated for this variable. As shown in Fig. 1B, a significant increase ($p = 0.046$) in 6-SMT was observed in the FOJ group (1,443.00 ± 596.00) compared to the OJ group (929.60 ± 518.80).

3.3. Effect of FOJ ingestion on antioxidant activity

The antioxidant activity of the urine samples was evaluated at 0, 2, 5, 10, 15, and 24 h after the consumption of OJ or FOJ using by three different approaches: TAC, FRAP and ORAC assays.

Fig. 2A shows the creatinine-corrected TAC values after OJ or FOJ consumption. TAC levels were significantly higher in the FOJ group at 2 h (97.67 ± 39.53 CRE, $p < 0.0001$), 5 h (100.06 ± 25.18 CRE, $p < 0.0001$), and 10 h (90.48 ± 34.20 CRE, $p = 0.0001$) post-ingestion compared to 0 h. A similar profile was observed in the OJ group (2 h: 88.13 ± 7.74 CRE, $p < 0.0001$; 5 h: 98.96 ± 34.65 CRE, $p < 0.0001$; 10 h: 71.99 ± 27.49 CRE, $p < 0.0001$). Although no significant differences were observed between the two experimental groups at any specific time point, the AUC of the TAC values during the experiment showed a significant increase ($p = 0.036$) in the FOJ group (1,421.00 ± 354.30) compared to the OJ group (1,138.00 ± 183.80) (Fig. 2B).

Fig. 3A shows the creatinine-corrected FRAP values after OJ or FOJ consumption. FRAP levels increased at 2, 5 and 10 h after consumption

Table 1
Quality parameters, bioactive compounds and antioxidant activity of orange juice (OJ) and fermented orange juice (FOJ). Results are expressed as means ± SD (n = 3). TA, titratable acidity; TSS, total soluble solids; ORAC, oxygen radical absorbance capacity.

Parameters	OJ	FOJ	p-value
pH	3.48 ± 0.20	3.48 ± 0.03	n.s.
TA (g citric acid/L)	8.48 ± 0.02	8.60 ± 0.01	n.s.
TSS (°Brix)	11.00 ± 0.50	9.01 ± 0.14	n.s.
% Pulp	12.00 ± 2.00	5.65 ± 0.22	< 0.05
Alcohol (% v/v)	0.00	0.90 ± 0.15	–
Ascorbic acid (mg/L)	423 ± 1.80	197 ± 6.80	< 0.0001
Total sugars (g/L)	78.2 ± 5.64	47.9 ± 4.10	< 0.0001
Reducing sugars (g/L)	48.5 ± 3.63	20.3 ± 2.40	< 0.0001
Non-reducing sugars (g/L)	29.7 ± 2.01	27.1 ± 2.61	n.s.
Melatonin (ng/mL)	0.185 ± 0.033	0.192 ± 0.072	n.s.
Tryptophan (mg/L)	13.8 ± 2.7	25.6 ± 3.3	< 0.05
Total (poly)phenols (μmol/L)	659.0 ± 10.5	835.7 ± 37.6	< 0.01
ORAC (μmol/L)	6361 ± 261	6353 ± 307	n.s.

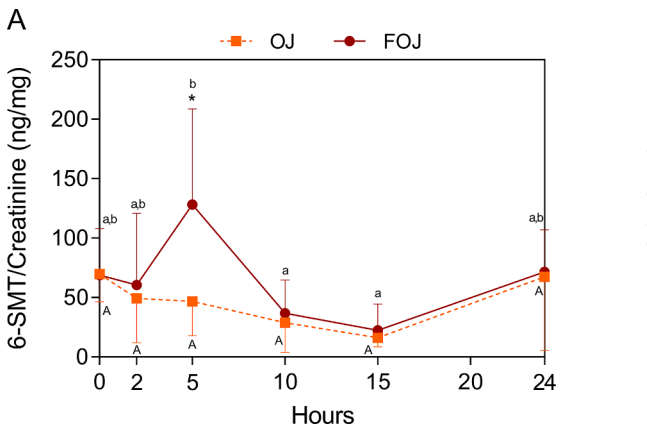

Fig. 1. 6-sulfatoxymelatonin (6-SMT)/creatinine concentration in urine sample throughout the experiment (0 – 24 h) (A). Area under the curve (AUC) of 6-SMT/creatinine concentration (B). Data represent the mean ± standard deviation. *, $p \leq 0.05$ between OJ and FOJ groups. Different capital and lowercase letters indicate the statistical difference ($p \leq 0.05$) between the OJ and FOJ groups at different time points, respectively. OJ, orange juice; FOJ, fermented OJ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

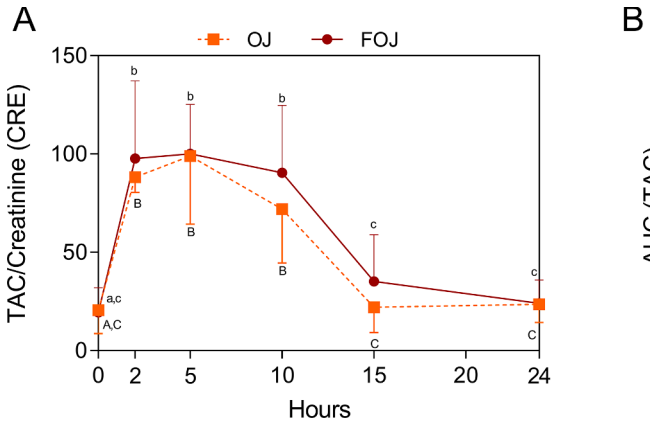

Fig. 2. Total antioxidant capacity (TAC)/creatinine concentration in the urine sample throughout the entire experiment (0 – 24 h) (A). Area under the curve (AUC) of the TAC/creatinine concentration (B). Data represent the mean ± standard deviation. *, $p \leq 0.05$ between the OJ and FOJ groups. Different capital and lowercase letters indicate the statistical difference ($p \leq 0.05$) between the OJ and FOJ groups at different time points, respectively. OJ, orange juice; FOJ, fermented OJ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

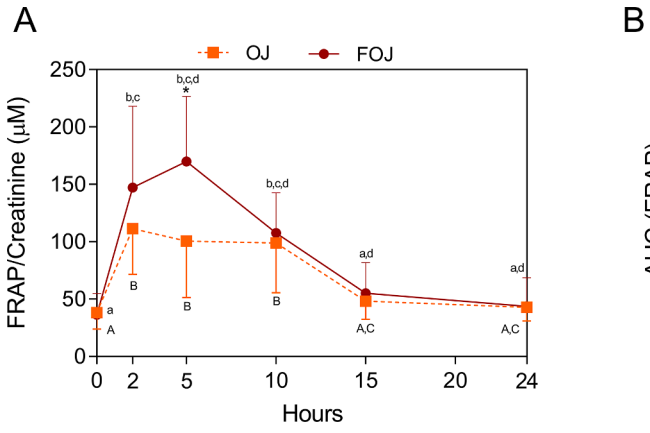

Fig. 3. Ferric reducing antioxidant power (FRAP)/creatinine concentration in urine sample throughout the experiment (0 – 24 h) (A). Area under the curve (AUC) of FRAP/creatinine concentration (B). Data represent the mean ± standard deviation. *, $p \leq 0.05$ between OJ and FOJ groups. Different capital and lowercase letters indicate the statistical difference ($p \leq 0.05$) between the OJ and FOJ groups at different time points, respectively. OJ, orange juice; FOJ, fermented OJ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of both OJ and FOJ with respect to the baseline (OJ; 0 h: 38.16 ± 14.29 μM; 2 h: 111.28 ± 39.67 μM, $p = 0.003$; 5 h: 100.46 ± 49.08 μM, $p = 0.0043$; 10 h: 98.86 ± 43.30 μM, $p = 0.0041$; FOJ; 0 h: 36.30 ± 18.62 μM; 2 h: 147.26 ± 70.84 μM, $p < 0.0001$; 5 h: 169.88 ± 56.75 μM, $p < 0.0001$; 10 h: 107.50 ± 35.17 μM, $p = 0.0262$).

A significant difference in FRAP levels was observed between the FOJ (169.88 ± 56.75 μM) and OJ (100.46 ± 49.08 μM) groups after 5 h of ingestion ($p = 0.0281$). The AUC calculation of the FRAP values confirmed the increase ($p = 0.018$) of FRAP in the FOJ group (2,253.00 ± 696.20) compared to the OJ group (1,546.00 ± 373.80) (Fig. 3B).

Fig. 4A shows the creatinine-corrected ORAC levels after OJ or FOJ consumption. The ORAC values were significantly higher in the FOJ group at 2 h (171.67 ± 59.80 μM, $p = 0.0043$) and 5 h (176.68 ± 76.12 μM, $p = 0.0013$) after ingestion compared to 0 h. A similar pattern was observed in the OJ group (5 h: 150.80 ± 44.30 μM, $p = 0.040$; 10 h: 147.46 ± 79.33 μM, $p = 0.042$). A significant difference was observed between the two experimental groups after 2 h of ingestion ($p = 0.0152$). The AUC calculation showed a significant increase in the ORAC values in the FOJ group (3,032.00 ± 620.90) compared to the OJ group (2,044.00 ± 951.90) during the experiment ($p = 0.029$) (Fig. 4B).

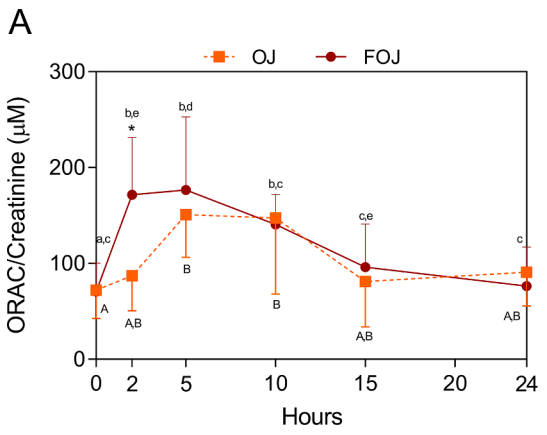

Fig. 4. Oxygen radical absorbance capacity (ORAC)/creatinine concentration in urine sample throughout the entire experiment (0 – 24 h) (A). Area under the curve (AUC) of ORAC/creatinine concentration (B). Data represent the mean ± standard deviation. *, $p \leq 0.05$ between the OJ and FOJ groups. Different capital and lowercase letters indicate the statistical difference ($p \leq 0.05$) between the OJ and FOJ groups at different time points, respectively. OJ, orange juice; FOJ, fermented OJ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Correlation between urine 6-SMT levels and antioxidant activity values

To evaluate the relationship between antioxidant activity and 6-SMT levels after OJ or FOJ consumption, a Spearman correlation between both parameters was applied. Table 2 shows a positive correlation between TAC and 6-SMT levels after FOJ ingestion, whereas no correlation was observed in subjects who consumed OJ.

4. Discussion

The present study highlights, for the first time, the beneficial effects of the alcoholic fermentation process on the bioavailability of MLT. In particular, FOJ consumption in healthy volunteers increased the presence of the 6-SMT metabolite as well as the antioxidant activity in the urine of the subjects. In addition, we described that the higher levels of 6-SMT were correlated with the increased total antioxidant activity, indicating a relationship between MLT content in FOJ and antioxidant effects.

The initial nutritional analysis of OJ and FOJ showed that the MLT content of OJ was 0.185 ng/mL which was very similar to 0.150 ng/g obtained by other authors (considering the density of orange juice) (Johns et al., 2013; Sae-Teaw et al., 2013). In contrast, although a previous report showed an increase in MLT levels after alcoholic fermentation (Fernández-Pachón et al., 2014), in the present study the MLT content in FOJ was similar to that of OJ. This loss of MLT could be explained by the thermal processing carried out to obtain the pasteurized FOJ (at 80 °C for 30 s) used in this study. Similarly, a loss of flavonoids and carotenoids content was previously observed in pasteurized FOJ (Escudero-López et al., 2016). Based on these results, Pranil, Moongngarm & Loypimai (2020) showed significant thermal degradation of MLT in aqueous solutions subjected to temperatures of 90 °C to 60 °C.

Although no differences in MLT concentration were found between OJ and FOJ, it is important to note that the 6-SMT levels were higher in the volunteers after ingesting FOJ when compared to the levels after ingesting OJ. Various processing techniques can improve the bioavailability of MLT in relation to the food matrix. It is important to note that although MLT possesses amphiphilic properties that allow it to dissolve in both alcohol and water, it has better solubility in alcohol than in water (Elia, Azoulay, & Zeiri, 2012; Garcia-Moreno, Calvo, & Maldonado, 2013). Due to this reason, the accessibility of MLT from the watery environment of the gastrointestinal tract is very difficult, resulting in a low bioavailability of MLT (Vlachou et al., 2017). The moderate alcohol content of FOJ (0.9 % v/v) can also increase the hydrophilicity of the medium and thus improve the bioaccessibility and bioavailability of MLT. In addition, although the influence of the fiber content of foods on the bioavailability of MLT has not previously been studied, an inverse relationship between the fiber content of fruits and the degree of intestinal absorption of other phytochemicals has been reported (Aschoff et al., 2015; Stevens-Barrón et al., 2019). A lower soluble fiber content (owing to the reduction of the pulp by 6 %) in FOJ compared to OJ was obtained. Therefore, the higher levels of 6-SMT in urine observed in the volunteers that ingested FOJ compared to OJ may be attributable to the fermentation-related factors that enhance the bioaccessibility of this compound.

On the other hand, in a previous study, our group described that the tryptophan concentration in FOJ (25.60 ± 3.3 mg/L) is significantly higher than in OJ (13.80 ± 2.7 mg/L) (Cerrillo et al., 2015). The amino acid tryptophan is the precursor of all 5-methoxytryptamines (or indoleamines), including MLT (Chattoraj, Liu, Zhang, Huang, & Borjigin, 2009). Previous studies have shown that the consumption of foods with a high tryptophan content improves the MLT synthesis in the gastrointestinal tract, increasing the plasmatic MLT concentration (Bubenik, 2002; Sánchez et al., 2008). In fact, although MLT can be synthesized by various body tissues, the intestine produces 400 times more MLT than the pineal gland (Chen, Fichna, Bashashati, Li, & Storr, 2011). Therefore, it is plausible that higher concentrations of tryptophan present in FOJ can lead to higher levels of MLT, and subsequent urine 6-SMT, after FOJ ingestion compared to OJ.

Consequently, the properties of FOJ derived from its processing, including the presence of ethanol, the tryptophan content, and the lower soluble fiber content could be associated with a better bioavailability of MLT.

It is well known that oxidative stress is responsible for the onset and progression of many chronic pathologies including neurodegenerative diseases, cardiovascular diseases, cancer, diabetes, and obesity, which are the leading cause of mortality worldwide, with approximately 35 million deaths each year (World Health Organization, 2020). Patients with hypertension, diabetes, or cancer have been shown to have low levels of urinary TAC (Choromańska et al., 2021; Peluso & Raguzzini, 2016). The selected antioxidant activity assays (TAC, FRAP, and ORAC assays) are based on different chemical reactions, solvents, and radical species, and the antioxidant activity results may differ among the measurement methods. Thus, the antioxidant activity must be evaluated by several methods of diverse reactions, being the three chosen methods widely used in biological fluids (Apak, Özyürek, Güçlü, & Çapanoğlu,

Table 2
Spearman correlation coefficients (r) between urine 6-SMT (6-sulfatoxymelatonin) levels and TAC (Total Antioxidant Capacity) values after OJ (orange juice) or FOJ (fermented orange juice) consumption.

	OJ		FOJ	
	r	p-value	r	p-value
TAC vs 6-SMT	− 0.01356	0.950	0.5648	0.005

2016; Cruz-Chamorro et al., 2021; Hoseini et al., 2019; Olszewska et al., 2020). Despite this limitation, an appropriate use of these techniques can be useful for the interpretation of oxidative status. In our study, we have used more than one technique to assess the antioxidant status, as recommended by the literature. Furthermore, as it is shown in the results, there are no significant differences in the first measurement (time 0) of ORAC, FRAP, and TAC before FOJ and OJ consumption and after the two weeks of washing, so we can rule out the existence of any external variable that has generated changes in oxidative status during the two weeks of washing. Even during the 24 h, we avoided any nutritional aspect that provides changes in the oxidative status. In addition, our objective was only to evaluate changes in urine antioxidant status after consumption of FOJ or OJ. For this reason, the limitation based on variations between laboratories (use of techniques, extraction, etc.) is not applicable in this study. By removing these limitations, the use of these methods provides a complete picture of antioxidant status cheaply and in a single measure (Pellegrini, Vitaglione, Granato, & Fogliano, 2020). Of interest is that the use of urine can avoid some limitations of plasma (invasive collection procedure, handling and storage conditions, pre-treatments, etc.). In fact, albumin and urea, two major endogenous antioxidant activity reactants, have been shown to not affect urine antioxidant activity (Ialongo, 2017). Thus, in the present study, the total antioxidant status of urine was measured using TAC, FRAP and ORAC assays with the aim of evaluating the potential of free-radical scavenging.

In general, FOJ consumption generated a better urinary antioxidant status in volunteers compared to that generated by OJ intake. Interestingly, a positive and significant correlation was observed between total antioxidant activity and 6-SMT levels in the urine of participants who ingested FOJ, but not OJ, was observed. Although correlation does not necessarily imply causation, these data point to a relationship between the increased bioavailability of MLT generated by the alcoholic fermentation process and the increase in antioxidant activity observed in the urine of volunteers after FOJ intake. This idea is also supported by the lower levels of glutathione, an important antioxidant molecule, in the FOJ (4.64 ± 0.70 mg/L) compared to OJ (10.90 ± 0.60 mg/L), previously described (Cerrillo et al., 2015). In accordance with this fact, OJ contains twice ascorbic acid than FOJ, lost during the pasteurization process, and it also contains 0.9 % (v/v) alcohol, which can induce oxidative stress (Comporti et al., 2010).

In a previous study the absorption, metabolism, and urinary excretion of OJ and FOJ (poly)phenols upon consumption, in acute conditions, were assessed (Castello et al., 2020). Thus, the results showed that the fermentation does not influence the pharmacokinetic and urinary excretion parameters of (poly)phenol metabolites. A total of 23 metabolites were identified and quantified in urine over 24 h after consumption of OJ or FOJ. Compounds identified included ten phase II-conjugated flavanones, between hesperetin, naringenin, and flavanone derivatives, three hydroxycinnamates, two hydroxyphenylpropanoic acids, one hydroxyphenylacetic acid, four hydroxybenzoic acids, and three hippurates. The excretion profiles of metabolites in urine were similar between OJ and FOJ for all the compounds ($p > 0.05$), and cumulative urinary excretion of individuals, classes, and total phenolic metabolites (0 – 24 h) was equal between treatments (total phenolic metabolites: 194,93 ± 32,93 μmol [OJ] vs 193,87 ± 34,34 μmol [FOJ]; $p > 0.05$). In addition, although FOJ presented higher value ($p < 0.05$) of total (poly)phenols (835,7 ± 37,6 μmol/L) than OJ (659,0 ± 10,5μmol/L) (Table 1), this difference did not modify the insights related to the influence of the food matrix on the bioavailability of orange juice (poly)phenols. Recovery rate (bioavailability estimated), considering the 24 h cumulative excretion of (poly)phenol metabolites in urine, and the amount of (poly)phenols, provided by the OJ and the FOJ, were not statistically different between them (OJ: 59.2 ± 10.0 %; FOJ: 46.4 ± 8.2 %, $p < 0.05$). Therefore, the significant increase in the antioxidant activity of the urine sample after the consumption of FOJ would be mainly due to the increase in MLT bioavailability.

Investigation with larger sample size is necessary to add more robustness to the results. In addition, due to the fact that MLT is considered a pleiotropic molecule that participates in several physiological processes (Carrillo-Vico, Lardone, Álvarez-Sánchez, Rodríguez-Rodríguez, & Guerrero, 2013), future studies will be aimed to evaluate the effect of the FOJ on other aspects of health.

5. Conclusions

This study describes an enhancement of urinary 6-SMT in human subjects after 500 mL of FOJ ingestion compared to OJ, which shows an interesting advantage of the fermentation process to increase the bioavailability of MLT. The present study shows for the first time the antioxidant properties of FOJ consumption in healthy humans by increasing the bioavailability of MLT, a molecule with potent antioxidant effects. Because MLT, in addition to having antioxidant properties, is a multifaceted molecule that participates in several physiological processes, FOJ could be a new functional food with potential positive effect on the maintenance of health status and prevention of chronic diseases.

6. Ethics Statement

The study was approved by the Clinical Research Ethics Committees of Virgen del Rocío Hospital and Pablo de Olavide University, Seville, Spain (IEC 2013PI/022).

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105325.

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