IMPACT OF PROCESSING ON THE NUTRITIONAL AND FUNCTIONAL VALUE OF MANDARIN JUICE

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Abstract

Background: Although phenolic compounds have a role in the health benefits of fruit juice consumption, little is known about the effect of processing on their bioaccessibility. The release of phenolic compounds from the food matrix during digestion is an important pre-requisite for their effectiveness within the human body, so it is fundamental to identify technological treatments able to preserve not only the concentration of phytochemicals but also their bioaccessibility. In this study we investigated the impact of high-pressure homogenization (HPH), alone and in the presence of 100g kg^{-1} trehalose or *Lactobacillus salivarius*, on bioaccessibility of flavonoids in mandarin juice. In addition, digested mandarin juices were supplemented to liver cultured cells in basal and stressed condition to evaluate their protective effect in a biological system.

Results: HPH reduced the concentration of total phenolics and main flavonoids but increased their bioaccessibility after *in vitro* digestion ($p<0.001$). In basal condition, supplementation with all digested juices significantly reduced intracellular reactive oxygen species (ROS) concentration ($p<0.001$). Thiobarbituric acid reactive substances concentration in the medium was also reduced by supplementation with HPH-treated juices. Although pre-treatment with juices did not completely counteract the applied oxidative stress it preserved cell viability, and cells pre-treated with juices submitted to HPH in the presence of probiotics showed the lowest ROS concentration.

Conclusion: Our study represents an important step ahead in the evaluation of the impact of processing on the nutritional and functional value of food, which cannot simply be assessed based on chemical composition.

Keywords: mandarin juice, flavonoids, oxidative stress, cultured cells, *in vitro* digestion

1. Introduction

Epidemiological studies suggest that diets rich in fruits and vegetables are related to a lower incidence of several chronic diseases [1]. Fruit juices retain most of the nutritional characteristics of the raw material from which they are extracted [2] and they could represent a good strategy to increase fruit consumption improving the human diet. Consumption of fruit juices is increasing, mainly due to their convenience, and in many Countries national Dietary Guidelines indicate them as a possible substitute of one out of the five recommended daily portions of fruit and vegetable. In addition to their intrinsic nutritional characteristics, fruit juices can be a way to convey functional ingredients such as probiotics [3, 4], which may also ameliorate the sensory aspect of the juice.

Although the main determinants of the nutritional value of juices are the type and quality of raw fruits, processing has an important role as well. Beside the decrease in the concentration of micronutrients and phytochemicals [5, 6], processing may cause plant matrix disruption and cell cluster disintegration, so increasing the bioaccessibility of nutrients and phytochemicals, i.e. their release from the food matrix [7].

Technologically strategies are often applied to improve the organoleptic characteristics and to increase the shelf-life of fruit juices. High pressure homogenization (HPH) is widely used in the production of fruit juice-based beverages to improve viscosity, color, shelf-life, stability of the pulp, and to increase polyphenols bioaccessibility [8-10]. Trehalose addition is also common, since it stabilizes the juice suspension through the interaction with cloud compounds so exerting a protective effect on various technological processes [11].

Using mandarin juice (MJ) as model system, in this work we evaluated the impact of HPH processing on total antioxidant activity (TAC) and flavonoid concentration, profile and bioaccessibility. MJ is predominantly composed of water, has a low energy density and contains a range of key nutrients such as ascorbic acid, flavonoids, minerals, and phytochemicals [12, 13]. The major phytochemicals are phenolic compounds, a large group of secondary plant metabolites with an aromatic ring bearing one or more hydroxyl substituents, possessing antioxidant activity [14]. HPH was applied alone or in the presence of trehalose or *L. salivarius* spp. *Salivarius.*

To evaluate the biologic effect of MJ supplementation, *in vitro* digested juices were supplemented to cultured liver cells (HepG2 cells) in basal condition and before applying an exogenous oxidative stress. The effect of supplementation was verified by measuring cell viability, intracellular concentration of reactive oxygen species (ROS) and reduced glutathione (GSH) and thiobarbituric acid reactive substances (TBARS) concentration in the media.

2. Materials and methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin and Dulbecco's Phosphate-Buffered Saline (DPBS) were purchased from Lonza (Milan, Italy). 1-propanol was supplied by Carlo Erba (Milan, Italy). All other chemicals were purchased from Sigma-Aldrich (Milan, Italy) and were of the highest analytical grade.

2.2. MJ preparation

MJs were prepared as previously described in [15]. Briefly, ortanique fruit, a hybrid of tangerine and sweet orange (*Citrus sinensis* x *Citrus reticulata*) was provided by a local cooperative in Benaguacil (Valencia, Spain), and sent to the Department of Agriculture and Food Sciences, University of Bologna, Cesena (Italy). Fruits were immediately washed with tap water, drained and squeezed in an industrial extractor with finger cups (Exzel, Luzzysa; El Puig, Valencia, Spain). Raw juice was centrifuged (3645 g, 5 min) at 4°C (Beckman Coulter Avanti TM J-25, Milan, Italy) and the low pulp juice was then pasteurized at 63°C for 15 s with a pasteurizer Qb8-4 (Roboqbo, Bologna, Italy) for microbial inactivation.

The pasteurized juice (pMJ) was then submitted to three different technological processes: i. homogenization at 20 MPa (HMJ); ii. homogenization at 20 MPa of mandarin juice that contained trehalose in proportion 100g kg^{-1} of juice (HMJ+Tr); iii. homogenization at 20 MPa with 8 Log CFU/ml of *Lactobacillus salivarius* CECT 4063 (HMJ+Ls).

2.3. MJ chemical composition, total antioxidant capacity, total phenolic and flavonoid content

In pMJ, total soluble solids were measured as Brix degree with a digital refractometer (Pal-1; Atago Co., Ltd., Tokyo, Japan) and expressed as g soluble solids kg^{-1} liquid phase. Total titratable acidity was assessed by titration with 0.1N NaOH and expressed g citric acid kg^{-1} liquid phase. Maturity index was calculated by dividing soluble solids content to total titratable acidity.

Total antioxidant capacity (TAC) was measured evaluating the capacity of antioxidant molecules in the sample to reduce the radical cation of 2,2'-azino-bis-(3-ethylbenzothiazoline-6- sulfonic acid) (ABTS•+), and it was expressed as mmol of Trolox equivalents (TE) L^{-1} [16].

Total phenolic content (TPC) was determined as reported in Di Nunzio *et al.* [17] with slight modifications. Briefly, 45 µL of water were first pipetted into each well of a 96-wells plate. Then, 5 µL of sample and 25 µL of Folin-Ciocalteau reagent (1:1 in water, v/v) were added. After 5 min shaking, 25 µL of 200 g Na_2CO_3 kg^{-1} water and 100 µL of water were added to the mixture. The absorbance was measured after 60 min at 750 nm with a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland). Results were expressed as g gallic acid equivalent (GAE) L^{-1}.

Flavonoid content was determined as described in Betoret *et al.* [18] using an HPLC LC-1500 (Jasco, Carpi, MO, Italy) with a diode array detector (DAD) and filled with a C18 reversed-phase column (150 x 4.60 mm, Phenomenex Kinetex® 5U C18 100). Briefly, 30 ml of sample, previously filtered using a Whatman grade 1 filter, was passed through a Sep-Pack C18 cartridge. The cartridge was eluted with 5 ml of water:acetonitrile in proportion 4:6 (v/v). The resulting sample was filtered using a nylon membrane filter with a pore diameter of 0.45 µm. The HPLC system was operated in gradient at a flow rate of 1 ml/min using water:tetrahydrofuran (solvent A) and acetonitrile:tetrahydrofuran (solvent B) as the mobile phases. Flavonoids were detected at a wavelength of 280 nm and expressed as mg kg^{-1}.

2.4. In vitro digestion

MJs were *in vitro* digested according to the INFOGEST standardized protocol [19] as described in Valli et al. [20]. Each juice was digested in duplicate and the resulting final digested solutions were centrifuged at 50,000 g for 15 min. Supernatants were filtered with 0.2 µm membranes, and an aliquot was sequentially ultrafiltered with Amicon Ultra at 3 kDa of molecular weight cut-off (EMD Millipore, MA, US) in order to obtain solutions containing compounds small enough (<3KDa) to be potentially absorbed through the intestinal mucosa. Duplicate digested solutions were mixed and frozen until experiments.

To evaluate bioacessibility, flavonoid content was assessed in 0.2 µm filtered, digested MJ as described above.

2.5. HepG2 cells culture and supplementation

HepG2 cells were grown in DMEM with 10% (v/v) fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin, and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Once a week cells were split 1:20 into a new flask, and culture medium was changed every 48 h [21]. Cells were seeded in 6-well or 12-well plates at the concentration of 1×10^6 cells mL^{-1}. Cell

144 counting was carried out using the TC20™ Automated Cell Counter (Bio-Rad Laboratories;
145 Hercules, CA, US). After 24 h (75–80% confluence) cells were incubated with serum-free DMEM
146 containing the different <3KDa digested samples. Concentration for cell supplementation was
147 determined in preliminary experiments assessing cytotoxicity (data not shown). The highest
148 concentration (100 µL mL^{-1}) not causing any cytotoxic effect was used for experiments. To avoid
149 interference due to vehicle, some cells (unsupplemented, US) received a corresponding amount of a
150 solution obtained from a "blank" digestion, that is an *in vitro* digestion performed without the
151 addition of any food.
152 In some experiments, 24 h after supplementation cells were washed twice with warm DPBS and
153 exposed for 1 h to 4mM H_2O_2 in Earle's Balanced Salt Solution (EBSS) (116mM NaCl, 5.4mM
154 KCl, 0.8mM NaH_2PO_4, 26mM $NaHCO_3$, 2.38mM $CaCl_2$, 0.39mM $MgSO_4$) to cause an oxidative
155 stress [22].
156
157 *2.6. Cell viability*
158
159 Cell viability was measured using the 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide
160 (MTT) colorimetric assay [23] using a Tecan Infinite F200 microplate reader (Tecan, Männedorf,
161 Switzerland), and it was expressed as percent of corresponding control cells.
162
163 *2.7. Intracellular ROS concentration*
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165 Intracellular ROS concentration was determined spectrofluorimetrically based on the capacity of
166 reduced non fluorescent 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to penetrate the cell
167 membrane and being enzymatically hydrolyzed by intracellular esterases to the reduced non-
168 fluorescent reduced 2′,7′-dichlorofluorescein (DCFH). DCFH is rapidly oxidized to the highly
169 fluorescent DCF proportionally to ROS concentration in the sample. Briefly, cells were washed
170 twice with cold DPBS, lysed with 1 mL of cold Nonidet P-40 (2.5g kg^{-1} in DPBS), incubated on ice
171 under shaking for 30 min and centrifuged at 14,000g for 15 min [24]. DCF fluorescence intensity was
172 detected (λ_{ex} = 485 nm, λ_{em} = 535 nm) using a Tecan Infinite F200 microplate reader (Tecan,
173 Männedorf, Switzerland), normalized for protein content in the sample and expressed as percent
174 value of corresponding US cells.
175
176 *2.8. TBARS concentration*
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Concentration of TBARS, the end-products of lipid peroxidation, was evaluated in DMEM and EBSS as previously reported [25]. Briefly, DMEM or EBSS were centrifuged at 400g for 3 min, and 100 µL were added to a mixture containing 100 µL of TCA (300 g kg^{-1} in 0.25N HCl), 100 µL of thiobarbituric acid (TBA) (7.5 g kg^{-1} in 0.25N HCl), and 3 µL of BHT (10 g kg^{-1} in ethanol). The mixture was heated for 10 min in a boiling water bath, allowed to cool, and the TBA adducts were detected fluorimetrically (λ_{ex} = 535 nm, λ_{em} = 595 nm). TBARS level was normalized for mg of proteins in each well and expressed as percent value of corresponding US cells.

2.9. GSH content

Cells were lysed with 500 µL of cold Nonidet P-40 (2.5g kg^{-1} in DPBS), incubated for 30 min on ice under shaking, and centrifuged at 14,000g for 15 min. One hundred microliters of the supernatant were incubated with 50 µL DPBS and 50 µL of reagent buffer (160mM sodium phosphate, 4mM EDTA, 40g kg^{-1} SDS and 500µM DTNB) for 30 min. GSH was measured spectrophotometrically by reading the absorbance of the newly formed 5-thio-2-nitrobenzoic acid at 415 nm [26]. The obtained results were compared to the concentration-response curve of standard GSH solutions, normalized for protein content in the sample and expressed as GSH mg^{-1} protein.

2.10. Protein content

Cells were washed with cold DPBS, lysed with 500 µL of cold Nonidet P-40 (2.5g kg^{-1} in DPBS), incubated on ice with shaking for 30 min and centrifuged at 14,000g for 15 min. Supernatants were collected and protein content was determined by the Comassie assay using BSA as standard, as previously described [27] .

2.11. Statistical analysis

Statistical analysis was by the one-way ANOVA with Tukey's Multiple Comparison Tests. All analysis were performed in three replications of the experimental design.

3. Results

Soluble solid content, total titratable acidity, and maturity index of pMJ are reported in Tab. 1.

Although HPH significantly decreased TPC, particularly when applied in the presence of trehalose (Fig. 1A), it did not modify TAC in any condition (Fig. 1B).

The content of the main flavonoids in MJ, hesperidin, naruritin and didymin, was significantly decreased by HPH (Tab. 2).

In all digested samples, hesperidin, narirutin and didymin content was lower than in the corresponding juice, and it was influenced by the previous technological treatment. Bioaccessibility, i.e. percent release from the food matrix [28] was significantly higher after HPH than in pMJ (Tab. 3).

Supplementation with the different juices did not modify either cell viability (Fig. 2A) or GSH content (Fig. 2B). On the contrary, all supplementations significantly reduced intracellular ROS concentration (Fig. 2C). TBARS concentration in the medium was also reduced by supplementation with HPH-treated juices (Fig. 2D).

In US cells, the exposure to 4mM H_2O_2 caused a significant decrease in cell viability and intracellular GSH level, and a significant increase in intracellular ROS concentration and TBARS level in the medium. Pre-treatment of cells with MJ appeared protective toward oxidative stress, although to different extent. Supplementation with all juices counteracted the reduction of cell viability (Fig. 3A), and HMJ+Tr also reduced the decrease of intracellular GSH content (Fig. 3B). In addition, the increase of ROS concentration was significantly lower in cells pre-treated with HMJ and in HMJ+Ls than US ones (Fig. 3C).

4. Discussion

HPH processing significantly decreased TPC and flavonoid content of pMJ, probably by forces and temperature stresses created in the homogenization valve during the treatment. Hesperidin and didymin degradation were partially prevented by the addition of 100g kg^{-1} trehalose. This could be related to the stabilization of the juice cloud. Trehalose interacts and forms complexes with bioactive compounds, and its protective effect has been deeply documented also for other molecules as anthocyanins [29-31].

Despite the decreased concentration of phenolics, TAC was not affected by the processing. Citrus fruits contain high concentration of antioxidant compounds as vitamins A, C and E, coumarins, carotenoids and others, which contribute to the overall TAC at different extent [32]. In particular, vitamin C accounts for 65–100% of the antioxidant potential of beverages derived from citrus fruit [33, 34] and many studies indicated that its concentration is not modified by HPH treatment [30, 35, 36]. In addition, HPH can increase carotenoid availability due to the disruption of cells and membranes [37]. The invariance in TAC of MJ with HPH treatment could be explained by the compensatory action

of other antioxidant such as ascorbic acid. It has been proved previously that the homogenization pressures at 20 MPa with 100g kg^{-1} of trehalose content and probiotic microorganisms did not affect the vitamin C content [30,31].

As previously reported [38], in pMJ hesperidin, naruritin and didymin accounted for almost the 90% of total flavonoids. Their lower concentration in the digested fractions than in the corresponding not digested juices clearly indicated that these molecules were only partially released from the food matrix. Interestingly, HPH had a positive effect on flavonoid bioaccessibility. This could be related to the reduction of the particle size of the juice [15] that facilitates the release of bioactives from the matrix. The further increase of narirutin and didymin bioaccessibility observed applying HPH in the presence of *L. salivarius* could be due to modification of molecular interactions between the flavonoids and the food matrix. Dietary fibers act as a carrier of dietary antioxidants [39], and probiotics may metabolically regulate the release of some phenolic compounds linked to fibers [40].

Cell supplementation in basal condition confirmed the protective effect of citrus juice against ROS generation and lipid oxidation [41, 42]. It is conceivable that the stronger effect of HPH treated juices than pMJ on TBARS formation was related to the increased bioaccessibility of flavonoids and possibly other bioactives.

Independent of pre-treatment with MJ, cell exposure to H_2O_2 caused a significant decrease of GSH concentration and a significant increase of ROS concentration and TBARS level. We hypothesize that the induced oxidative stress was too strong to be completely counteracted by MJ. However, supplementation with digested juices preserved cell viability and HMJ and HMJ+Ls pre-treated cells showed the lowest ROS concentration among stressed cells. Our data confirm results by Cilla *et al*. [43], who evidenced that pre-incubation with the bioaccessible fraction of citrus pulp may protect Caco-2 cells against H_2O_2-induced oxidative stress preserving cell viability, mitochondrial membrane potential and cellular reduced status.

5. Conclusion

Using pMJ as model fruit juice, in this study we evidenced that, beside reducing TPC and flavonoid content, HPH treatment of MJ did not modify TAC and increased flavonoid bioaccessibility after *in vitro* digestion.

Regardless HPH treatment, in basal condition supplementation with all juices counteracted ROS formation and lipid peroxidation in liver cells. Although pre-treatment of cells with MJ did not completely counteract the effect of the oxidative stimulus, HMJ + Ls appeared the most protective juice. Although the overall effect of supplementation is the result of the synergistic action of many

different components, the highest protection by HMJ+Ls could be in part accounted to the observed highest flavonoid bioaccessibility. Addition of probiotics to juices could therefore represent not only a strategy for administering functional ingredients but also an effective way to increase accessibility of bioactive molecules.

Herein reported results highlight the impact of technological processing on bioaccessibility of active components, and their possible ultimate effect on food functionality. Notably, the use of <3KDa digested fractions for cell supplementation allowed us close mimicking the *in vivo* condition and considering a real food in spite of discrete food-derived molecules and/or extracts. Although the study has limitation since the impact of the microbiota on bioactive bioavailability was not considered, it represents an important step ahead in the evaluation of the nutritional and functional value of food, which cannot simply be assessed based on chemical composition [44, 45].

Conflict of interest

The authors declare no conflicts of interest. The funding sponsors had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

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Author contributions

Mattia Di Nunzio: Conceptualization, Formal analysis, Investigation, Methodology, Data curation, Writing - original draft. Ester Betoret: Formal analysis, Investigation, Methodology, Data Curation, Funding acquisition, Project administration. Annalisa Taccari: Formal analysis, Investigation, Methodology, Data curation. Marco Dalla Rosa: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. Alessandra Bordoni: Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing.

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453 **Table 1.**

454 Soluble solid content, total titratable acidity and maturity index of pasteurized mandarin juice.
455 Results are means ± SD of three replicates.

Soluble solids content (g kg^{-1})	136 ± 0.2
Total titratable acidity (mg kg^{-1})	24.7 ± 0.2
Maturity index	5.5 ± 0.02

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458 **Table 2.**

459 Hesperidin, narirutin and didymin content of mandarin juice. Flavonoids content is expressed as mg
460 kg^{-1}. Data are means ± SD of three replicates. Statistical analysis was by one-way ANOVA
461 ($p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least
462 $p<0.05$).

Flavonoids (mg kg^{-1})	**pMJ**	**HMJ**	**HMJ+Tr**	**HMJ+Ls**
Hesperidin	139.4 ± 0.4^{a}	88.5 ± 0.9^{c}	98.0 ± 0.5^{b}	88.8 ± 0.9^{c}
Narirutin	129.5 ± 1.4^{a}	82.4 ± 8.7^{b}	83.9 ± 0.9^{b}	83.6 ± 8.4^{b}
Didymin	24.5 ± 0.4^{a}	15.7 ± 0.0^{c}	17.2 ± 0.1^{b}	15.1 ± 0.1^{c}

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Table 3.

Hesperidin, narirutin and didymin concentration in digested mandarin juice and bioaccessibility. Flavonoids content in digested MJ is expressed as mg kg^{-1}. Bioaccessibility was calculated as [flavonoid] after digestion/[flavonoid] before digestion x 100. Data are means ± SD of three replicates. Statistical analysis was by one-way ANOVA (digested samples: hesperidin $p<0.01$, narirutin $p<0.001$, didymin n.s.; bioaccessibility: $p<0.001$) with Tukey's post-hoc test. Different letters in the same row indicate significant differences (at least $p<0.05$).

	pMJ	**HMJ**	**HMJ+Tr**	**HMJ+Ls**
Concentration (mg kg^{-1})				
Hesperidin	1.6 ± 0.3^{b}	2.1 ± 0.1^{ab}	2.3 ± 0.1^{a}	2.4 ± 0.2^{a}
Narirutin	3.4 ± 0.1^{b}	2.5 ± 0.0^{c}	2.8 ± 0.1^{c}	4.9 ± 0.2^{a}
Didymin	0.8 ± 0.1^{a}	0.7 ± 0.0^{a}	0.7 ± 0.1^{a}	0.8 ± 0.1^{a}
Bioaccessibility (%)				
Hesperidin	9.0 ± 1.6^{b}	19.0 ± 0.9^{a}	18.8 ± 0.4^{a}	21.8 ± 2.0^{a}
Narirutin	20.7 ± 0.6^{c}	24.4 ± 0.4^{b}	26.5 ± 1.0^{b}	46.6 ± 2.4^{a}
Didymin	24.9 ± 1.9^{c}	35.5 ± 2.0^{b}	33.0 ± 2.7^{b}	44.2 ± 3.7^{a}

Figure Captions

Figure 1. TPC (A) and TAC (B) of mandarin juice.

TPC (panel A) is expressed as g of Gallic Acid Equivalents (GAE) L^{-1}; TAC is expressed as mmol of Trolox Equivalents (TE) L^{-1}. Data are means ± SD. Statistical analysis was by one-way ANOVA (A: $p<0.001$; B: n.s.;) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

Figure 2. Cell viability (A), GSH content (B), and level of ROS (C) and TBARS (D) in unsupplemented (US) and supplemented cells.

Cell viability (panel A), ROS (panel C) and TBARS level (panel D) are expressed as % of the corresponding value in US cells (assigned as 100%). GSH content (panel B) is expressed as nmol mg^{-1} protein. All data are means ± SD of at least six samples derived from three independent experiments. Statistical analysis was by the one-way ANOVA (C and D, $p<0.001$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).

Figure 3. Cell viability (A), GSH content (B), and level of ROS (C) and TBARS (D) in unsupplemented (US) and supplemented cells.

Cell viability (panel A), ROS (panel C) and TBARS level (panel D) are expressed as % of the corresponding value in US cells in basal conditions (assigned as 100%). GSH content (panel B) is expressed as nmol mg^{-1} protein. All data are means ± SD of at least six samples derived from three independent experiments. Statistical analysis was by the one-way ANOVA (A, C and D, $p<0.001$; B, $p<0.01$) with Tukey's post-hoc test. Different letters indicate significant differences (at least $p<0.05$).