npj | science of food

Article

Published in partnership with Beijing Technology and Business University & International Union of Food Science and Technology

https://doi.org/10.1038/s41538-025-00531-7

Arginine Maillard reaction products recovered damaged immune cells

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Instant noodle has been an initiating point for food industry in many countries, and it continues to be a beloved convenient food in the world. However, some products cause discomfort after consumption despite full compliance in both ingredients and processing methods. It has not only resulted in serious consumer misunderstanding but also affect the industry as well. A simple solution possibly lies in nowhere but its soup which uses Maillard reaction products as flavoring agents. Arginine-Glucose Maillard reaction products (Arg-Glc MRPs) have been demonstrated to be antioxidant and anti-inflammatory, which can be useful to remove food caused discomfort. Here we show that Arg-Glc MRPs neither produced significant cytotoxicity nor caused oxidative stress in RAW264.7 macrophages. In theF APPH-induced oxidative damage model, they remarkably reduced cytoplasmic ROS and $O_2^{\cdot-}$, and restored the polarization of cytoplasmic membrane potential and mitochondrial membrane potential while enhancing the phagocytic function of normal cells and oxidatively damaged cells. Those in vitro results indicate that the usage of Arg-Glc MRPs in the soup can be a promising solution to discomfort problems.

Instant noodle is a phenomenon invention in the history of convenient food. It has been a seed that is instrumental for the formation and development of food processing industry in countries all over Asia and other parts of the world. The surprise and awe of first experience of enjoying it remain vivid in many consumers' memory for its novelty in convenience and prominent taste beyond expectation in comparison with home-made noodle. In fact, the earliest versions of instant noodle are marketed as snack food in Japan, China and many countries, which echoes its huge past impact on generations of consumers.

Despite its glorious past, instant noodle has been falling off consumer's favor countries with the increase of per capital income rise[1,2]. For example, instant noodle sale has plateaued since 2004 in China regardless of the explosive increase of its most frequent consumption points such as a long trains journey[3]. Meanwhile criticism and claims of its health risks have been flooded all over the media, vilifying it in the top list of ultra processed food to avoid. Facing the current difficult situation, the industry defends itself with the existing science of perfect compliance in terms of both ingredients and processing methods, with limited influence to defuse public concerns. The association of instant noodle consumption with many metabolic syndromes is a subject for discussion, far from anything conclusive[4–6]. Yet one thing real is that some of instant noodle can cause discomfort along the alimentary tract. A striking image captured by a capsule camera of indigestibility of instant noodle has shocked the public, which resonates well consumer's personal body memory[7]. As a matter of fact, long journey train passengers seldom take two consecutive meals of instant noodle, which explains the reason behind a flat sale against the explosive increase in the sales opportunity in China. It is too premature to associate food cause discomfort with health concerns, but it is true that the discomfort can reinforce the virilized image of instant noodle. Nutrition science and toxicology are the sciences supporting regulating food ingredients and process compliances but offer no clues to issues like food caused discomfort. Insistence on incomplete food science can legitimately silence complaints but not really help the industry in real sense because it is the food caused discomfort (FCD) that is naturally discouraging the consumption of instant noodle. It is imperative for scientists to advance food science to provide solutions to remove FCD in the best interest of both consumers and the industry.

In face of whatever challenges science is the only way out. If there is a trust in science there is always a way. With a deep faith in science, a simple solution can be found in nowhere but the making of instant noodle soup which incorporates Maillard reaction products as a key flavoring agent. The Maillard reaction is the chemistry that endows heat processed food with attractive attributes like color, aroma and flavors. Much less known and underexploited functions of versatile MRPs is their potent antioxidant and anti-inflammatory activity, which is a simple and effective antidotes against

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FCD which is typical expression of oxidative stress or inflammation of the mucosa layer at different parts of the alimentary tract. We developed Arginine-Glucose Maillard reaction products (Arg-Glc MRPs), that has been used in instant noodle to completely eliminating the reflux problem[8].

Of 20 amino acids, arginine has not been the most used amino acid in MRPs production because of its cost. The major consideration to use arginine to produce MRPs is that its potent antioxidant and anti-inflammatory activity[9,10]. Its Maillard reaction products with glucose usually could not exhaust both reactants regardless varying reaction condition control like reactants ratio and other conditions. It is therefore a mixture of MRPs and residual reactants. It is neither necessary nor easy to remove residual reactants from the mixture. As a basic amino acid, arginine has a high reactivity. Arg-Glc MRPs have also been demonstrated to suppress the formation of acrylamide in the Maillard reaction[11]. Metformin containing two guanidine groups is most famous antidiabetic drug, it would not be unreasonable to expect similar benefit on guanidine-containing arginine which is a food ingredient.

RAW264.7 mouse macrophage cell line, constructed from the ascites of BALB/c mice transformed with Abelson leukemia virus[12], is a well-established canonical system in immunology and cell biology because of its multitasking ability. It has been widely used for evaluating the biological effects of natural food components, e.g. evaluating innate immune-enhancing abilities of alkaloids, glycosides, flavonoids, phenolic compounds, fatty acids, and glycoproteins, etc.[13,14]. It is used as a cellular model in this study to investigate the antioxidant effects of Arg-Glc MRPs preparations in order to eventually exploit MRPs to their full potential in food application.

Results

Degree of the Maillard reaction and extracellular antioxidant capacity

The pH levels of Arg-Glc MRPs diminished as the reaction time increased (Fig. 1a). This decline could be attributed to the formation of organic acids, including acetic acids and formic, as well as the utilization of amino groups during the initial phase of the Maillard reaction[15,16].

The absorbance measurements at 294 nm and 420 nm serve as indicators for the intermediates and final products of the Maillard reaction, respectively[17,18]. As shown in Fig. 1b, c, the absorbance at both 294 nm and 420 nm increased significantly along with reaction time, indicating that the formation of reaction intermediates and final products was time-dependent[19]. Figure 1d shows the full range (250–800 nm) absorption spectra of Arg-Glc MRPs after heating for 0.5, 1, and 2 h, separately. And the Arg-Glc MRPs have the maximum absorption peak at 298 nm with an intensity of 0.592.

Figure 1e illustrates that the capacity of Arg-Glc MRPs to scavenge DPPH radicals steadily enhanced as the reaction time was extended. Meanwhile, the reducing power of MRPs (Fig. 1f), which is commonly employed to assess the antioxidative activity of Maillard-type conjugates, also gradually increased as the reaction proceeded. Both of the results above suggested that the MRPs exhibited a varying degree of extracellular antioxidant capacity according to their reaction time.

Effects of Arg-Glc MRPs on cell viability

Figure 2a indicates that the MTT assay showed no significant cytotoxicity of Arg-Glc MRPs towards RAW264.7 macrophages, with cell viability remaining above 90%. LDH, a cytosolic enzyme commonly recognized as a marker for cytotoxicity[20,21], was measured in the culture medium, revealing that treatment with Arg-Glc MRPs resulted in increased leakage of LDH (Fig. 2b). Therefore, Hoechst 33258 staining was utilized to examine alterations in cell morphology following treatment with Arg-Glc MRPs. The results showed no evidence of cell shrinkage or nuclear condensation (Fig. 2d), indicating that MRPs did not cause morphological damage and there was no significant change in the number of apoptotic cells (Fig. 2c). In summary, while treatment with Arg-Glc MRPs led to an increase in LDH leakage, which may indicate potential membrane damage, the absence of morphological changes and significant apoptosis suggests that these MRPs do not exert marked cytotoxic effects on RAW264.7 macrophages.

Effects of Arg-Glc MRPs on intracellular ROS and superoxide anion ($O_2^{\cdot-}$)

To study the regulatory effect of Arg-Glc MRPs on cellular redox status, DCFH-DA green fluorescence was used to analyze total ROS production in RAW264.7 macrophages, while DHE red fluorescence was used to evaluate the production of superoxide anion ($O_2^{\cdot-}$). Figure 3a illustrates that the addition of Arg-Glc MRPs prepared with different reaction times alone does

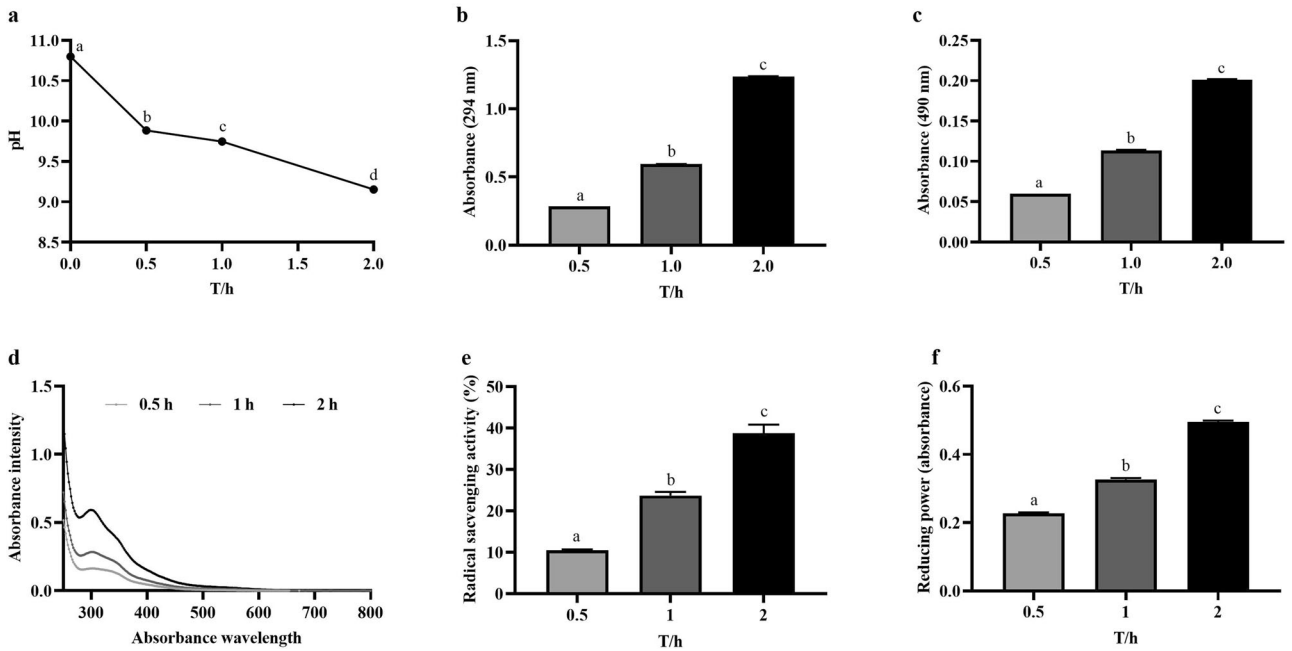

Fig. 1 | Arg-Glc Maillard reaction degree and extracellular antioxidant capacity. pH (**a**), absorbance at 294 nm (**b**), absorbance at 420 nm of the Arg-Glc MRPs (**c**); full range (250–800 nm) absorption spectra of Arg-Glc MRPs (**d**); the DPPH radical scavenging activity of Arg-Glc MRPs (**e**); the reducing power of Arg-Glc MRPs (**f**). The values are expressed as the mean ± SEM ($n = 3$); Different letters indicate significant differences at $p < 0.05$ by Tukey–Kramer's t-test.

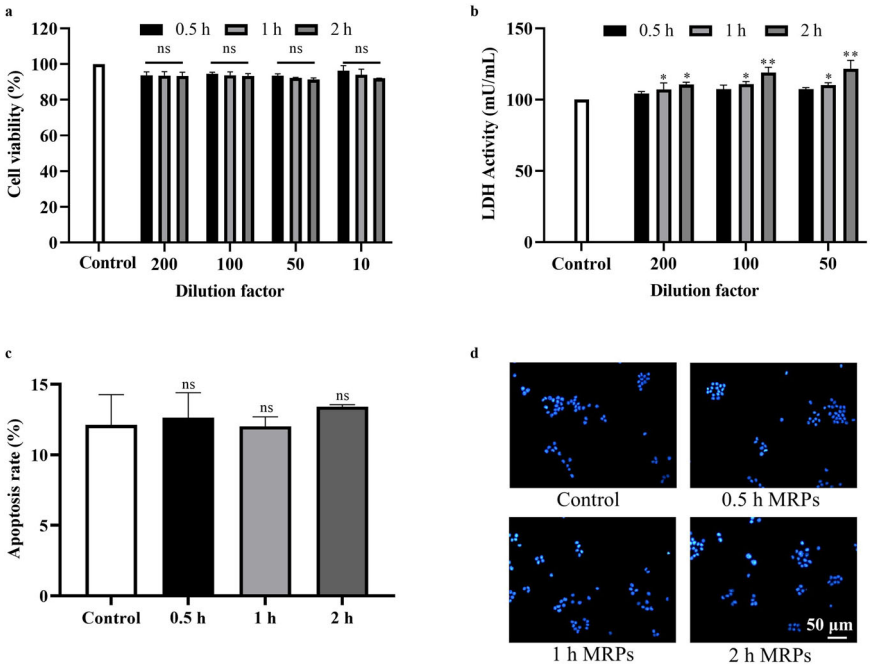

Fig. 2 | Effects of Arg-Glc MRPs on cell viability. Cell viability determined using the MTT assay (**a**); the activity of lactate dehydrogenase (**b**); quantification of apoptotic cells (MRPs was diluted with DMEM: 1:100, v/v) (**c**); image of cell nuclei labeled with Hoechst 33258 (**d**). The values are expressed as the mean ± SEM ($n = 3$); $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control by LSD test.

not affect the production of ROS, and no obvious change in green fluorescence intensity can be observed in the microscopic image (Fig.3b). Exposure to AAPH significantly increased intracellular fluorescence intensity (from 131.44 ± 10.28 in the control group to 252.17 ± 16.65), and its green fluorescence was significantly enhanced. The treatment with Arg-Glc MRPs effectively reduces the buildup of intracellular ROS that is triggered by AAPH. For example, after adding 0.5 h, 1 h and 2 h MRPs diluted 100 times, the fluorescence intensity decreased to 179.15 ± 6.77, 156.27 ± 8.17 and 116.214 ± 12.83 respectively, and the same results can be seen in the green fluorescence image, indicating that the longer the preparation time, the greater the protective effect against the increase in ROS caused by AAPH.

Knowing that the Arg-Glc MRPs are beneficial for total ROS scavenging, the production of superoxide anions($O_2^{\cdot-}$) in RAW264.7 macrophages was analyzed using DHE fluorescence analysis[22]. Arg-Glc MPPs treatment had no significant effect on $O_2^{\cdot-}$ content, but AAPH stimulation significantly increased $O_2^{\cdot-}$ production (from 24.00 ± 1.16 in the control group to 31.62 ± 1.39), and its red fluorescence intensity was significantly enhanced (Fig. 3c, d). Co-treatment with Arg-Glc MRPs effectively reduced the increase in $O_2^{\cdot-}$ caused by AAPH stimulation. These results suggest that Arg-Glc MRPs can regulate cellular redox status and inhibit hyperoxidation in RAW264.7 macrophages.

Intracellular antioxidant capacity of the Arg-Glc MRPs

The intracellular antioxidant capacity of Arg-Glc MRPs was assessed using the CAA method across selected concentrations and various reaction times. The kinetics of AAPH-induced oxidation on RAW264.7 macrophages is shown in Fig. 4a–c. Arg-Glc MRPs inhibited the rise in fluorescence due to DCF formation in a concentration-dependent manner. This effect is illustrated by the curves obtained from cells treated with MRPs prepared for 0.5 h (Fig. 4a), 1 h (Fig. 4b) and 2 h (Fig. 4c). The antioxidant capacity of the tested samples was calculated from the kinetic curves (expressed as CAA values in Fig. 4d). The results show that the intracellular antioxidant capacity increases with the extension of reaction time and exhibits concentration dependence, which is consistent with the results of DPPH radical scavenging ability.

Changes in cytoplasmic membrane potential

The Arg-Glc MRPs did not significantly influence the cytoplasmic membrane potential (MPs) of normal RAW264.7 macrophages (Fig. 5a), but counteracted the membrane hyperpolarization on AAPH-stimulated macrophages (Fig. 5b). When the free radical elicitor AAPH induced free radical damage on RAW264.7 macrophages, the MPs of macrophages dropped significantly within 2 h, indicated by the green fluorescence intensity of $DiBAC_4(3)$ probe (Fig. 5c). While the Arg-Glc MRPs and AAPH were co-incubated with macrophages, green fluorescence was partially restored, suggesting a counteraction of membrane hyperpolarization. The 2 h MRPs showed the highest capacity to restore membrane potential, suggesting that prolonging the Maillard reaction time could enhance this capacity.

Protective effects of Arg-Glc MRPs on mitochondrial function

Mitochondria are the primary source of ROS in cells, and regulating mitochondrial redox activity and MMP may be an effective strategy for improving mitochondrial dysfunction[23,24]. The Mito-SOX Red probe was used to study the effect of Arg-Glc MRPs on mitochondrial superoxide anion free radicals. As shown in Fig. 6a, in the presence of AAPH, the fluorescence intensity of Mito-HE on RAW264.7 macrophages decreased from 125.39 ± 9.35 to 80.87 ± 7.02, possibly due to AAPH-induced mitochondrial oxygen respiration dysfunction. However, after the addition of 0.5 h, 1 h and 2 h Arg-Glc MRPs diluted 100-fold, the fluorescence intensity increased to 131.018 ± 5.62, 162.44 ± 5.96 and 245.75 ± 20.28, respectively (Fig. 6b). The specific fluorescence image is shown in Fig. 6a. This suggests that co-incubation with both AAPH and Arg-Glc MRPs resulted in significantly counteracting the inhibition of mitochondrial oxygen respiration.

Treatment with AAPH can lead to mitochondrial dysfunction via various mechanisms, such as lipid peroxidation of the mitochondrial membrane, damage to mitochondrial DNA, and alterations in transmembrane potential[25,26]. As shown in Fig. 6c, AAPH led to a reduction of MMP on RAW264.7 macrophages, indicated by the decrease of red fluorescence intensity and the increase of green fluorescence intensity, which suggests the damage to MMP by intracellular ROS[27–29]. However, the addition of 2 h MRPs alone had no significant effect on the fluorescence of JC-1, but was able to repair ROS-induced MMP damage to varying degrees in AAPH-stimulated macrophages. As Fig. 6d shows that the relative fluorescence ratio increased from 3.34 ± 0.3 in the AAPH-stimulated group to 3.76 ± 0.16 after the addition of the Arg-Glc MRPs.

Changes in phagocytic ability of neutral red in RAW264.7 macrophages

Figure 7 shows that all tested Arg-Glc MRPs greatly enhance phagocytosis in normal macrophages or AAPH-stimulated macrophages. The Arg-Glc

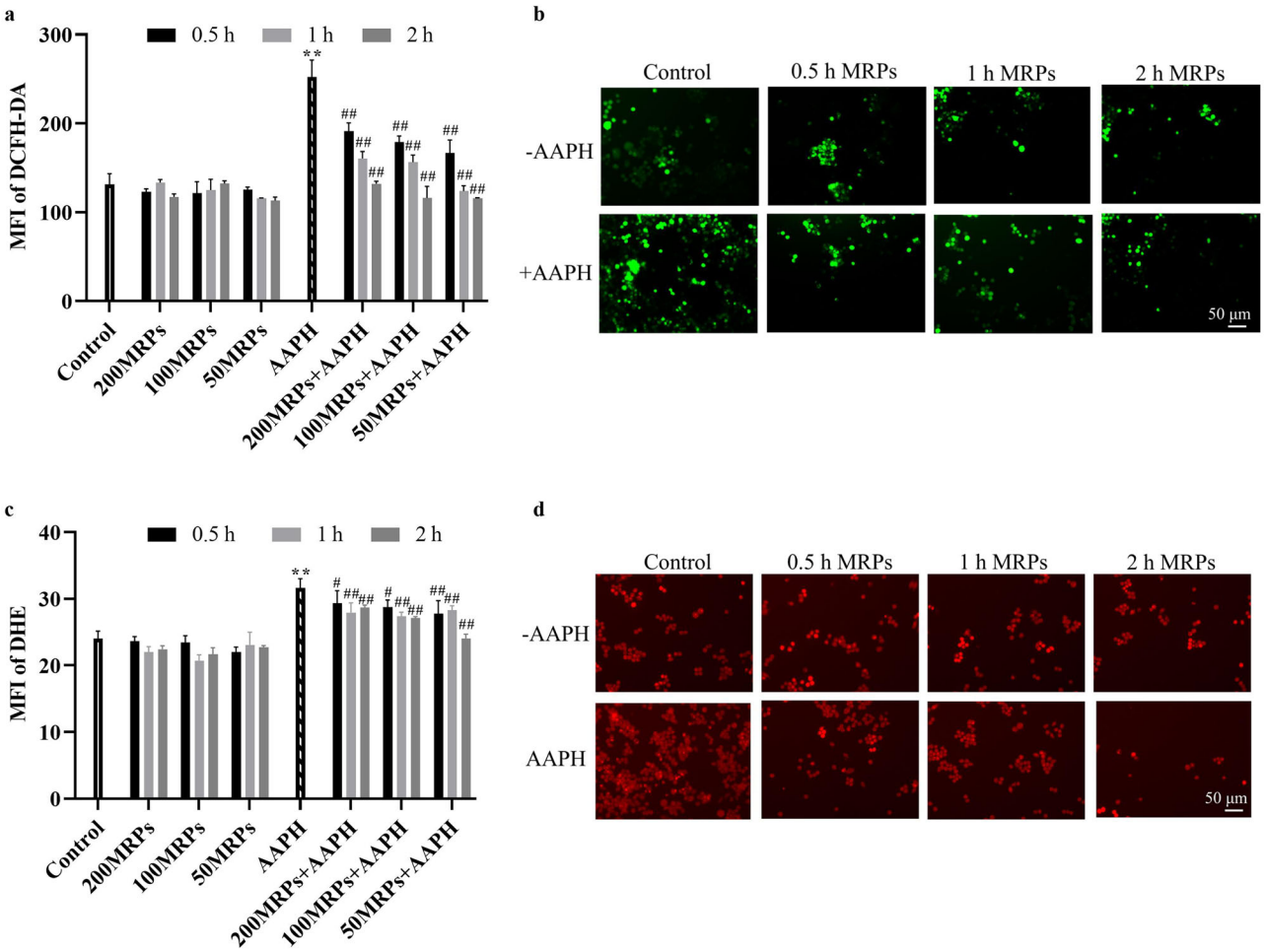

Fig. 3 | Effects of Arg-Glc MRPs on ROS and $O_2^{\cdot-}$ in RAW264.7 macrophages. Mean fluorescence intensity of DCFH-DA in cells (**a**); green fluorescence microscopy images indicate intracellular ROS content (**b**); mean fluorescence intensity of DHE in cells (**c**); red fluorescence microscopy images indicate intracellular $O_2^{\cdot-}$ content (**d**). In the experiment, the dilution factors of MRPs were 50, 100, and 200, with the fluorescence image showing the cells at a 100-fold dilution. The values are expressed as the mean ± SEM ($n = 3$); $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. AAPH, $^{\#\#}p < 0.01$ vs. AAPH by LSD test.

MRPs significantly enhanced the phagocytic activity of normal macrophages. After the challenge of AAPH, the phagocytic activity of macrophages was dramatically reduced from 100 to 52.49 ± 3.37, indicating an impairing effect caused by oxidative damage. Notably, engulfment of any Arg-Glc MRPs resulted in significantly enhancing the phagocytic capacity, therefore restoring the AAPH-suppressed phagocytosis.

Discussion

Beyond a simple source of nutrients, food directly or indirectly regulates various processes of the immune system[30,31]. The accompanying reactions during food processing, transportation and storage are often unavoidable; their impact on food quality and human health cannot be ignored. This study proposes that MRPs present in the food system can affect and possibly restore macrophages from oxidative stress-induced damage.

The redox imbalance may cause a series of oxidative pathological reactions, which could further affect metabolic function and induce various health problems[32]. The results showed that Arg-Glc MRPs exhibited significant in vitro and intracellular antioxidant activity. Specifically, the vitamin C equivalent of the DPPH free radical scavenging capacity of 2 h MRPs was 1.41 ± 0.08 mg Vc/mL (Table S1), which is significantly higher than that of organic green tea extract[33]. In terms of cellular antioxidant activity, the quercetin equivalent of 2 h MRP was 1.49 ± 0.03 μmol QE/mL (Table S2), which also had a high antioxidant level[34]. As reported in previous studies, MRPs have antioxidant activity[35], and the change of extracellular antioxidant activity of MRPs with heating time[36], while less attention has been paid to cellular redox balance. In vivo studies have found that MRPs can reduce the accumulation of ROS and promote cellular stress tolerance through the insulin/IGF-1 signaling pathway[37]. Given the high reactivity and transient nature of reactive oxygen species, along with the presence of various antioxidant mechanisms in biological systems and the specificity of fluorescent probes, we employed DCFH-DA and DHE probes for the selective detection of ROS and $O_2^{\cdot-}$ in living cells, respectively. Additionally, the MitoSOX Red fluorescent probe was utilized specifically to quantify superoxide anion levels within mitochondria. This approach aimed to investigate how Arg-Glc MRPs modulate the redox state of RAW264.7 macrophages[38,39]. The results showed that Arg-Glc MRPs can mitigate oxidative stress in AAPH-stimulated RAW264.7 macrophages and have good intracellular antioxidant capacity.

Macrophages are at the front line of the body's immune response and have excessive tolerance to endogenous or environmental ROS. Dysfunction of membrane potential, even slight abnormal changes, can greatly affect intracellular biological activities including inflammatory responses, thereby causing various diseases[40–42]. MRPs neither disturb the plasma nor mitochondrial membrane potential on RAW264.7 macrophages. In other words, exposure to Arg-Glc MRPs did not stimulate macrophages. The azo compound AAPH was utilized to produce peroxyl radicals, simulating the ROS challenges that macrophages might face in the gut microenvironment during the process of food consumption[43]. MRPs restore MP and MMP to normal resting levels, thus protecting macrophages and mitochondria from AAPH-induced peroxidation of free radicals, indicating their soothing effect

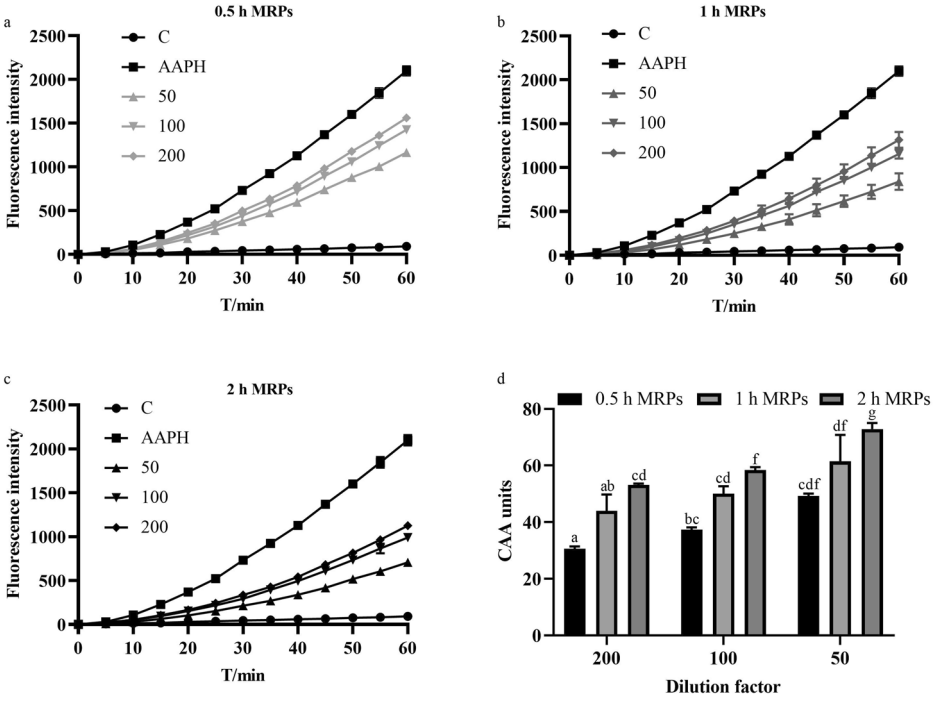

Fig. 4 | Cellular antioxidant activity (CAA) of Arg-Glc MRPs. Peroxyl radical-induced oxidation of DCFH to DCF in RAW264.7 macrophages and the inhibition of oxidation by Arg-Glc MRPs (diluted with DMEM: 1:50, 1:100, 1:200, v/v) over time (**a**–**c**); cellular antioxidant activity (CAA) of Arg-Glc MRPs (**d**). The values are expressed as the mean ± SEM ($n = 3$); Different letters indicate significant differences at $p < 0.05$ by Tukey–Kramer's t-test.

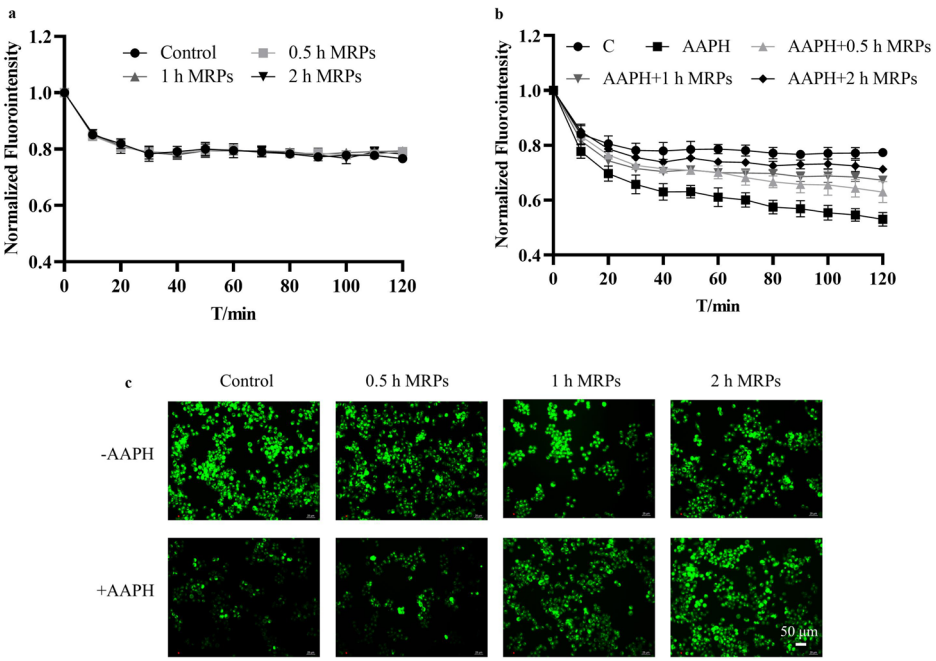

Fig. 5 | Effects of Arg-Glc MRPs on the membrane potential (MPs) of RAW264.7 macrophages. Cells MP-dependent fluorescence traces (**a**, **b**); green fluorescence micrographs, taken with a Leica DMI3000B inverted microscope, excitation at 515 nm and emission at 490 nm (**c**).

on stimulated macrophages. The plasma membrane, a primary target for ROS that induce lipid peroxidation, is affected by oxidative stress, which leads to lipid peroxidation and results in decreased membrane fluidity and altered physiological functions[44,45]. Although the regulatory mechanism of MRPs on cell membrane potential is unclear, a previous study evaluated the possible effect of Arg-Glc MRPs on phospholipid bilayers, and the results showed that Arg-Glc MRPs can stabilize lipid bilayers by Lamellar/non-lamellar structure formation of simian immunodeficiency virus (SIV) peptides modulates membrane biological events[46]. This result may have certain relevance and provide a basis for regulating some membrane-related biological events, but whether the regulation of membrane potential is related to the activity of lipid phase transition still needs further study.

Furthermore, our results indicate that Arg-Glc MRPs can significantly restore the phagocytic function of macrophages challenged by oxidative stress, which is a component of the immune response to foreign substances[47,48]. This finding partially reflects the role of MRPs in enhancing immunity; however, the relationship between MRPs and macrophage immune activation requires further elucidation through chemical or biological methods.

Arg-Glc MRPs was demonstrated to possess repairing effects on the damaged RAW264.7 cell model and its addition remarkably mitigated the reflux caused by instant noodles[8], which implies that the damage of macrophages may be associated with the reflux though other influences need to be taken into consideration. It is reported that Macrophages in the gastric

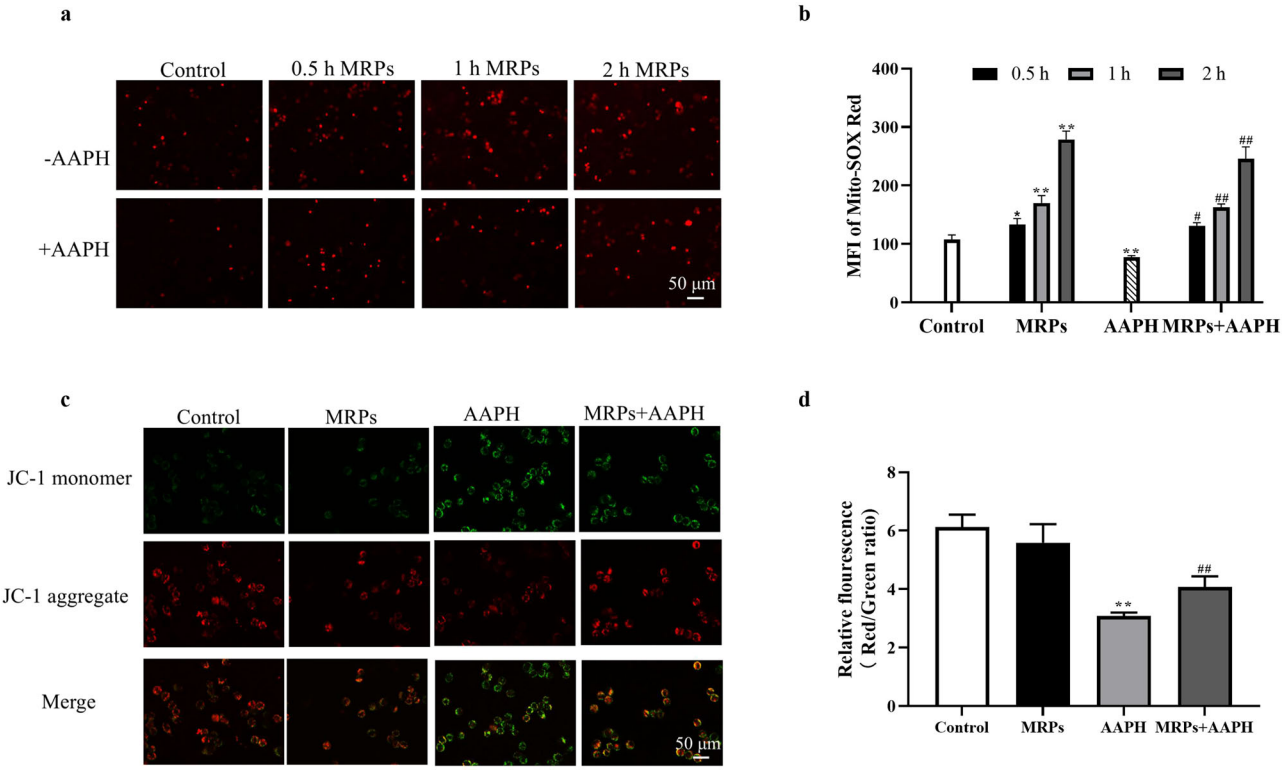

Fig. 6 | Effects of Arg-Glc MRPs on mitochondrial function in RAW264.7 macrophages. Red fluorescence image of mitochondrial superoxide levels in cells (**a**); mean fluorescence intensity of Mito-SOX Red in cells (**b**); confocal microscopy images of cells labeled with JC-1, JC-1 aggregates are shown in red, JC-1 monomers are shown in green, and merge image combined aggregate and monomer signals (**c**); evaluation of MMP changes using JC-1 probe red/green fluorescence ratio (**d**). The values are expressed as the mean ± SEM ($n = 3$); $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. AAPH, $^{\#\#}p < 0.01$ vs. AAPH by LSD test.

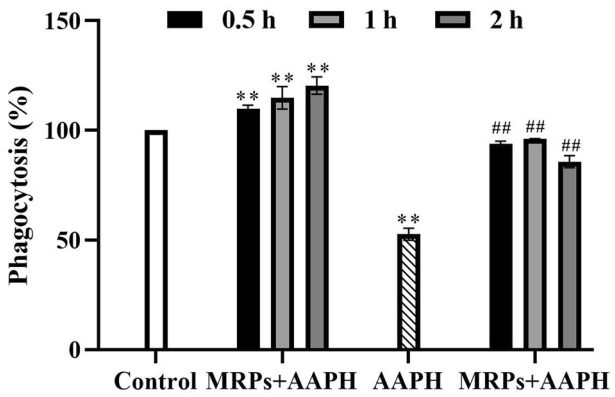

Fig. 7 | Effects of Arg-Glc MRPs on the phagocytosis of RAW264.7 macrophages. The values are expressed as the mean ± SEM ($n = 3$); $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. AAPH, $^{\#\#}p < 0.01$ vs. AAPH by LSD test.

mucosal immune system interact with macrophages in the muscularis capable of controlling peristaltic activity[49]. The damage of macrophages in the gastric lining compromises the peristaltic activity and resulted in a small pocket of undigested noodles, which was bounced back upon gastric movement as reflux. More investigation is required to understand the mechanism of arginine MRPs on cellular repairing. What is explicit is its prominence of in vivo antioxidant activity, which can be helpful in repairing the damaged macrophages. Components with in vivo antioxidant activity could potentially demonstrate effects on reducing food caused discomforts caused by mucosal stress at different sites with different degrees.

However, previous reports have suggested that MRPs, as a complex cocktail of chemicals, can have both beneficial and detrimental health effects, which are associated with the Maillard reaction process[50]. The harmful part mainly comes from the advanced glycation end products (AGEs), acrylamide (AM), heterocyclic amines (HCA), etc., produced by the Maillard reaction. Generally considered to be cytotoxic, research has found that both endogenous and exogenous AGEs can accumulate in tissues, activating macrophages and leading to the production of excessive inflammatory cytokines. This process can contribute to the development of several chronic diseases, including Alzheimer's disease, cancer, diabetes, Parkinson's disease, and chronic heart failure[51–53]. The Arg-Glc MRPs we prepared mainly contain early-stage products, which show positive effects on RAW264.7 macrophages, which may be mainly caused by melanoidins, reductones, and furan compounds in the MRPs. In subsequent studies, the intermediate and late-stage MRPs can be prepared by controlling the reaction conditions (such as changing the reaction time, temperature, pH, etc.) to investigate whether they have adverse or positive effects on RAW264.7 macrophages.

In addition, different amino acids and reducing sugars will generate MRPs with varying chemical properties and biological activities[54,55]. Arginine has previously been used as a substrate in the Maillard reactions[56,57], and Arg-Glc MRPs are the most abundant MRPs found in traditional Chinese herbal medicines, suggesting that it may have significant potential effects[46]. Arginine and glucose were selected as substrates to prepare MRPs, and this was used as an entry point to elucidate the influence of MRPs on macrophages. However, follow-up studies that target complex food Maillard reaction systems and are not limited to in vitro studies, but are based on experimental animals, may make the research more complete. The in vivo uptake process of food-derived MRPs should be much more complicated, the direct interaction between the Arg-Glc MRPs and macrophages demonstrated in this study is clear and may contribute to future studies of uptake, transport and modulation of immunity in vivo by Maillard reaction products, based on current studies conducted at the cellular level in vitro.

In this study, Arginine-Glucose Maillard reaction products with different reaction times all demonstrated no cytotoxicity on RAW264.7

macrophages but exhibited remarkable DPPH free radical scavenging and extracellular reducing abilities. They could all effectively quench intracellular free radicals caused by AAPH, and helped to restore cytoplasmic membrane potential (MPs) and mitochondrial membrane potential (MMP) of RAW264.7 macrophages damaged by AAPH. Moreover, all the Arg-Glc MRPs enhanced phagocytosis of macrophages. All those outstanding antioxidant and anti-inflammatory properties results provide a strong support for the Arg-Glc MRPs in food as a safe, effective and simple antidote against food caused discomfort.

Methods

Materials

L-Arginine and D-(+)-Glucose anhydrous were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). AAPH, $DiBAC_4(3)$, and MitoSOX™ Red mitochondrial superoxide indicator were obtained from Sigma (St. Louis, MO, USA.). DMEM, PBS, HBSS, JC-1 Dye, FBS, streptomycin sulfate and penicillin were obtained from Thermo Fisher Scientific (Waltham, MA, USA). MTT was provided by MCE Technology Co. (New Jersey, USA.). LDH cytotoxicity assay kit and mitochondrial permeability transition pore assay kit were purchased from Beyotime Biotechnology (Shanghai, China). All other chemicals and solvents used were of AR grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Preparation of Maillard reaction products

A 0.6 mol/L arginine aqueous solution is mixed with an equal amount of glucose, and then the mixture is subjected to heat treatment at 100 °C for processing times of 0.5 h, 1 h, and 2 h, respectively. After heating, the reactions were promptly halted by immersing the samples in an ice bath. The resultant solutions of Arg-Glc MRPs were then preserved at −30 °C for subsequent applications.

Measurements of pH and browning

The pH of the Arg-Glc MRPs was determined by a pH meter (METTLER TOLEDO, Zurich, Switzerland), which was standardized against buffer solutions at pH levels of 4.0, 7.0, and 10.0.

Arg-Glc MRPs were diluted to a ratio of 1:100 (v/v) with deionized water prior to absorbance measurements at 294 nm and 420 nm, conducted using a spectrophotometer (U-5100, HITACHI, Japan). Following this, Arg-Glc MRPs solutions were also diluted to a 1:1000 (v/v) ratio with deionized water, and the ultraviolet absorption spectra were recorded across the wavelength range of 250 nm to 800 nm through multiple spectrophotometric measurements.

Determination of extracellular antioxidant activities

The antioxidant activity of Arg-Glc MRPs diluted with deionised water at a ratio of 1:100 (v/v) in the extracellular environment was evaluated by quantifying DPPH radical scavenging activity (DPPH-RS)[58] and reducing power (RP) using previously reported experimental methods[59], where the DPPH radical scavenging activity was compared with vitamin C as a standard control. The reducing power assay is based on the reduction reaction of potassium ferrocyanide ($K_3[Fe(CN)_6]$) and is used to evaluate the antioxidant activity or reducing substance content of a sample by detecting the change in absorbance when Prussian blue ($Fe_4[Fe(CN)_6]_3$) is formed.

Cell viability (MTT test)

RAW264.7 macrophages were seeded in 96-well plates at a density of 1×10^4 cells/mL, and after reaching 80% confluence, MRPs (diluted with DMEM: 1:10, 1:50, 1:100, 1:200, v/v) were added and co-incubated for 24 h, respectively. Subsequently, Methylene blue solution was added for an additional 2.5 h incubation period at 37 °C. Afterward, the supernatant was aspirated and 150 μL of DMSO was added, which was then gently agitated to ensure thorough mixing. Subsequently, the optical density was assessed at 570 nm utilizing a microplate reader (FlexStation 3, Molecular Devices, USA).

Lactate dehydrogenase (LDH) release

Following a 24-h exposure to Arg-Glc MRPs (diluted with DMEM: 1:50, 1:100, 1:200, v/v), the release of lactate dehydrogenase (LDH) from RAW264.7 macrophages was evaluated using an LDH cytotoxicity detection kit, in accordance with the instructions provided by the manufacturer.

Hoechst 33258 staining

Following a 24-h exposure to Arg-Glc MRPs (diluted with DMEM: 1:100, v/v), the RAW264.7 macrophages were washed with HBSS and incubated with 3 μg/mL Hoechst 33258 for 10 min at 37 °C. Fluorescence micrographs were taken with an inverted microscope (DMI3000 B Inverted Microscope, Leica) connected to LAS (VERSION 4.2.0) software immediately after the fluorescence experiment. After that, the microscopic images were analyzed to enumerate the apoptotic nuclei characterized by condensed chromatin, and the count of these apoptotic nuclei was then calculated as a percentage relative to the total nuclei count.

Intracellular ROS measurement

After RAW264.7 macrophages were co-incubated with Arg-Glc MRPs for 6 h, the macrophages were washed and stained with 20 μM DCFH-DA for 25 min. The fluorescence intensity was measured (Ex = 485 nm, Em = 528 nm) and fluorescence images were taken as in the previous experiment.

Measurement of superoxide anion ($O_2^{\cdot-}$)

Intracellular $O_2^{\cdot-}$ levels were determined by $O_2^{\cdot-}$ specific fluorescent probe dihydroethidium (DHE). The RAW264.7 macrophages were stained with 5 μM DHE for 30 min, subsequent to a 6-h incubation with Arg-Glc MRPs. After washing twice with HBSS, the fluorescence intensity was recorded (Ex = 535 nm, Em = 610 nm), and fluorescence micrographs were captured as well.

Intracellular antioxidant capacity assay

The antioxidant capacity of Arg-Glc MRPs (diluted with DMEM: 1:50, 1:100, 1:200, v/v) on RAW264.7 macrophages was quantified by examining the percentage reduction in DCFH-DA fluorescence (Ex = 485 nm, Em = 538 nm). The procedure was conducted as previously described for the cellular antioxidant activity (CAA) assay[60], with quercetin and vitamin C serving as standard controls.

Cytoplasmic membrane potential measurement

The influence of Arg-Glc MRPs (diluted with DMEM: 1:100, v/v) on cell cytoplasmic membrane potential (MPs) of RAW264.7 macrophages was assessed using a methodology previously reported[61]. $DiBAC_4(3)$ fluorescence intensity changes were collected by a microplate reader for 120 min with an interval of 10 min at 37 °C. Fluorescence photomicrographs were taken immediately after fluorescence intensity determination.

Mitochondrial superoxide assay

After the cells in the 96-well plate reached high confluence, the original medium was removed. Then, 200 μL of Mito-SOX Red (2.5 μmol/L) was added, and the plate was kept at 37 °C for 10 min. Discarded the working solution, washed the cells twice with warm HBSS, added Arg-Glc MRPs (diluted with DMEM: 1:100, v/v), and incubated in a cell incubator for 2 h. Then the absorbances were recorded by a microplate reader (Ex = 486 nm, Em = 570 nm).

Mitochondrial membrane potential measurement

After 2 h of action of Arg-Glc MRPs (diluted with DMEM: 1:100, v/v), changes in mitochondrial membrane potential (MMP) were measured were detected with a JC-1 fluorescent probe as previously reported[20], and red JC-1 aggregates and green JC-1 monomers were observed using a laser scanning confocal microscope (Zeiss LSM 780, Carl Zeiss SAS, Germany). In addition, the fluorescence intensities of JC-1 aggregates and monomers in a 96-well plate were measured using a microplate reader.

Determination of the phagocytic capacity of macrophages

The effect of Arg-Glc MRPs (diluted with DMEM: 1:100, v/v) on phagocytosis of neutral red by macrophages was performed as previously reported[62].

Statistical analysis

The results from distinct replicates are expressed as the mean ± standard error of the mean (SEM). All analyses were performed using the SPSS Statistics version 23.0 (SPSS Inc., Chicago, IL, USA) software. All data graphs were generated using GraphPad Prism 9.0 (Origin Lab Corporation, Northampton, MA, USA). Statistical differences between the two groups were evaluated using Student's t test, while one-way analysis of variance (ANOVA), followed by LSD or Tukey–Kramer's t-test for post-hoc analysis, was employed to analyze the differences between multiple groups. Statistical significance was set at $p < 0.05$.

Data availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Received: 23 October 2024; Accepted: 18 July 2025;
Published online: 01 August 2025

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Acknowledgements

This research was supported by Fujian Provincial Natural Science Foundation of China (Grant No. 2024J01162 and No. 2024J01967) and the 2023 Fujian Provincial Young and Middle-aged Faculty Education and Research Program (Science and Technology) (Grant No. JAT231074).

Author contributions

Yanan Ding: data curation, methodology, writing—original draft. Pingfan Rao: project administration, writing—reviewing and editing, resources. Jianwu Zhou: data curation, investigation, validation. Huiqin Wang: data curation, investigation, formal analysis. Ruiyang Wang: data curation, investigation. Lijing Ke: investigation, validation. Guanzhen Gao: conceptualization, supervision, writing—reviewing and editing.

Competing interests

The authors declare no competing financial interest or non-financial interests. P.R. is Editor-in-Chief and L.K. is Associate Editor of npj Science of Food. P.R. and L.K. were not involved in the journal's review of, or decisions related to, this manuscript.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41538-025-00531-7.

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