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Spatial distribution of antioxidant activity in baguette and its modulation of proinflammatory cytokines in RAW264.7 macrophages

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Baguette is a globally acclaimed bakery staple, composed by a crispy crust and soft crumb, both containing Maillard reaction products (MRPs) with potential bioactivities. However, MRPs' impacts on the nutritional and health attributes of baguette, particularly in terms of cellular and biological functions, are yet to be clearly elucidated. This study chemically characterizes the crust and crumb of baguettes and investigates the influence of the Maillard reaction on baguette's nutritional profile, especially in the antioxidant and anti-inflammatory effects. The findings indicate an increase in browning intensity and advanced glycation end products (AGEs) from the baguette's interior to its exterior, alongside a significant rise in the antioxidant capacity of the crust, suggesting the Maillard reaction's role in boosting antioxidative properties. Both the crust and crumb demonstrated strong cytocompatibility with immune cells, capable of reducing cellular oxidative stress and regulating intracellular free radical levels. The crust effectively countered peroxyl radical-induced cell membrane hyperpolarization by 91% and completely neutralized the suppression of oxygen respiration in mitochondria, displaying higher efficacy than the crumb. In contrast, crumb extracts were more potent in inhibiting lipopolysaccharide-induced expression of proinflammatory cytokines, such as interleukins-1β (IL-1β) and IL-6, in macrophages. It could provide the fundamental data and cell-based approach for investigating the biological impacts of bread on immune responses, contributing to the refinement and supplementation of nutritional recommendations.

The baguette stands out as a globally renowned bread variety, distinguished by its distinctive features, including a crisp crust measuring 3–4 mm in thickness, an open and heterogeneous crumb cell structure with a rich, full-bodied flavor[1]. These defining characteristics were intricately linked to the Maillard reaction, a complex set of chemical processes occurring during dough baking[2]. While existing literature extensively underscores the influence of the Maillard reaction on shaping the sensory attributes of bread[3], particularly in terms of color and texture quality[4,5], a notable gap persists regarding a comprehensive understanding of how the Maillard reaction specifically impacts the biological effects of baked bread. This raises the intriguing question: Is there a biological mechanism behind our love for delectable food?

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During baking process, a complex cascade of nonenzymatic reactions, the so-called Maillard reaction, is taking place between carbonyl groups of reducing sugars and amino acids, peptides and amino groups of proteins[6]. The Maillard reaction produces a variety of bioactive compounds such as melanoidins[7,8]. Melanoidins, characterized as high molecular weight, brown, and nitrogenous compounds, are formed in the final stages of the Maillard reaction, with a variety of biological activities such as antioxidant[9]. The melanoidins identified in the bread crust demonstrate an individual-dependent influence on distinct microbial flora, suggesting a potential anti-inflammatory effect through the inhibition of enterobacteria[10,11]. Pronylated lysine, found in bread melanoidins, has been shown to act as monofunctional inducers of Glutathione S-transferases, serving as a functional parameter of antioxidant and chemopreventive activity[12]. The Maillard reaction, while also introducing certain adverse effects on food such as the formation of AGEs and acrylamide[6,13], can be effectively managed through the precise regulation of baking conditions[2].

A healthful dietary regimen invariably exerts significant influences on immune responses, consequently playing a role in the regulation of appetite[14]. It can be posited that a health-conscious diet, through its modulation of immune reactions, augments the consumers' preference and dependence on such dietary patterns. In the context of immunometabolic dysregulation, immune cells undergo oxidative stress in the presence of inflammatory cytokines, thereby impacting appetite[15]. Prior investigations have elucidated the commendable standing of traditional and prevalent health-promoting diets, exemplified by the esteemed reputation of porcine bone soup[16,17]. Notably, the nanoparticles derived from these diets exhibit efficacious modulation of immune cell activity within the gastrointestinal mucosa, consequently ameliorating cellular oxidative stress[18,19]. Analogous observations are discerned in nanoparticles rich in polyphenols, exemplified by those found in aged vinegar[20]. Given the substantial population of immune cells, particularly macrophages, within the human intestinal mucosa, bearing pivotal roles in inflammation and metabolic disorders[21], it is imperative to establish a comprehensive model elucidating the intricate interplay between baguette consumption and immune cells.

By elucidating variances in spatial antioxidant properties, this study aims to elucidate the significant role played by the Maillard reaction in augmenting the antioxidative activity and immunocompetence of baguette, and then enables to extend knowledge regarding bread, particularly baguette, bioactive properties as a key food matrix quite prevalent on human dietary consumption habits. Furthermore, this study is anticipated to make a meaningful contribution to the improvement and supplementation of nutritional guidelines for baked foods and healthy diets, providing insights into the intricate relationships among the Maillard reaction, sensory preferences and desires, and underlying biological mechanisms. Such exploration is essential for a more comprehensive understanding of the overall impact of dietary choices.

Results and discussion

Chemical composition

As shown in Table 1, the dry matter of baguette crust extracts (BCstE, 7.46 mg/mL) was significantly lower than that of baguette crumb extracts (BCmbE, 8.57 mg/mL). The BCstE exhibited a higher concentration of proteins (0.77 mg/mL) and phenolic compounds (38.47 μg/mL). In contrast, BCmbE contained a greater amount of carbohydrates (5.95 mg/mL) and triglycerides (4.00 mmol/L), along with a higher turbidity level (143.67 mAbs). This study determined that the main chemical components of baguettes were carbohydrates and proteins, consistent with literature reports[22]. However, the composition content was relatively lower compared to what was reported in other studies, possibly due to the use of an aqueous extraction method which might not have fully extracted the insoluble components in bread. The Maillard reaction had a significant impact on the chemical composition of both the crust and crumb aqueous extracts. For instance, during the high-temperature baking process, the Maillard reaction caused a significant increase in soluble phenolic compounds, accompanied by a notable reduction in insoluble phenolic compounds[23]. Baking significantly reduced the carbohydrate content in food, possibly because carbohydrates underwent reactions such as the Maillard reaction with proteins[24]. Therefore, further exploration of the variation in the Maillard reaction degree between the crust and crumb was crucial for understanding the differences in composition.

Browning degree

Table 2 revealed distinct chromatic differences between the bread crust and crumb post-baking. The red-green degree (a^*) and yellow-blue degree (b^*) of bread crust were higher than those of bread crumb, but the lightness value (L^*) of bread crust was lower than that of bread crumb. Furthermore, the yellowing index of bread crust was notably higher than that of bread crumb. As shown in Table 2 and Fig. 1, despite similar lightness values (L^*) and red-green degree (a^*) between BCstE (baguette crust extract) and BCmbE (baguette crumb extract), BCstE displayed a greater yellow-blue value (b^*) than BCmbE with a significant difference. The yellowing index (YI), used as a more specific color index of the Maillard reaction (MR) in processed foods. The yellowing index of BCstE (17.81) was higher than that of BCmbE (10.61) with a significant difference, and these experimental findings are consistent with those reported by Delgado-Andrade et al.[25]. The above results indicated that the browning degree of bread crust was significantly higher than that of bread crumb, emphasizing that the primary browning occurs in the crust due to intense heat processing.

Color changes in baked goods occur mostly in the later stages of the Maillard reaction. the research shows that the final stage of the Maillard reaction products was darker in color than the early low molecular weight Maillard reaction products[5]. Because a series of reactions can occur during the heating process, such as cyclization, dehydration, rearrangement, isomerization and polymerization, and the final formation of nitrogenous brown copolymer or polymer (melanoidins)[7,8]. Comparing the color and browning intensity of crust and crumb extracts underscores that the Maillard reaction is more pronounced in the crust.

Content of fluorescent AGEs

Table 3 revealed that three types of fluorescent advanced glycation end products (AGEs)—specifically, glycosylated collagen, pentosidine, and derivatives of pyrrole and imidazole – were detected in both the bread crust and crumb. Notably, the concentrations of these AGEs were higher in the bread crust compared to the crumb. AGEs are a complex group of compounds that are formed through Maillard reaction, a nonenzymatic reaction between reducing sugars and free amino groups[26]. Within a certain range, the accumulation of AGEs is proportional to the degree of Maillard reaction, highlighting that Maillard reaction occurred, both in bread crust and bread crumb, being more prevalent in the bread crust as well as in its extract.

The extraction efficiency of AGEs from both bread crust and crumb was relatively low, with yields of 16–19% for glycosylated collagen, 20–27% for pentosidine, and 24–25% for derivatives of pyrrole and imidazole, which

Table 1 | Chemical profile of the bread crust and crumb extracts

Sample	Dry Matter (mg/mL)	Protein (mg/mL)	Carbohydrate (mg/mL)	Triglyceride (mmol/L)	Total phenol (μg/mL)	Turbidity (mAbs)
BCstE	7.46 ± 0.07^b	0.77 ± 0.03^a	5.36 ± 0.12^b	3.67 ± 0.15^b	38.47 ± 0.79^a	74.00 ± 1.00^b
BCmbE	8.57 ± 0.05^a	0.43 ± 0.02^b	5.95 ± 0.06^a	4.00 ± 0.02^a	25.29 ± 0.40^b	143.67 ± 2.08^a

Reported values are the mean ± SD ($n = 3$). Values bearing different superscripts in the same column are significantly different ($p < 0.05$).

were 4 to 5 times lower compared to their original levels in the bread crust and crumb as detailed in Table 4. This discrepancy aligns with previously observed differences, where the concentration of fluorescent Maillard reaction products in western breakfast cereal foods was up to 10 times higher than in their corresponding extracts[27], highlighting that extraction method may influence the AGE yield. Moreover, digestion of bread crust powder by streptomycin proteinase E solution may also affect the total fluorescent AGEs recovered in digested extract[28], which may explain the difference between the extraction yield of fluorescent AGEs in the food matrix bread (crumb and crust) and its extracts. While the fluorescence determination method does not capture certain AGEs such as carboxymethyl-lysine, carboxyethyl-lysine, and pyrrolidine[29,30], among others, it effectively highlights the presence of AGEs in Baguette and illustrates the variation in their distribution from crust to crumb.

Table 2 | Chromatic value and YI of the bread crust and crumb

Sample	CIELab color			Yellowing index (YI)
	L*	a*	b*	
BCst	57.50 ± 0.80^{b}	10.47 ± 1.00^{a}	34.86 ± 0.58^{a}	86.61 ± 0.24^{a}
BCmb	66.43 ± 2.13^{a}	-1.74 ± 0.08^{c}	15.69 ± 0.23^{b}	33.75 ± 0.59^{b}
BCstE	65.89 ± 0.98^{a}	-1.37 ± 0.07^{b}	8.21 ± 0.02^{c}	17.81 ± 0.32^{c}
BCmbE	65.08 ± 0.18^{a}	-0.50 ± 0.01^{b}	4.83 ± 0.03^{d}	10.61 ± 0.10^{d}

Reported values are the mean ± SD (n = 3). Values bearing different superscripts in the same column are significantly different (p < 0.05).

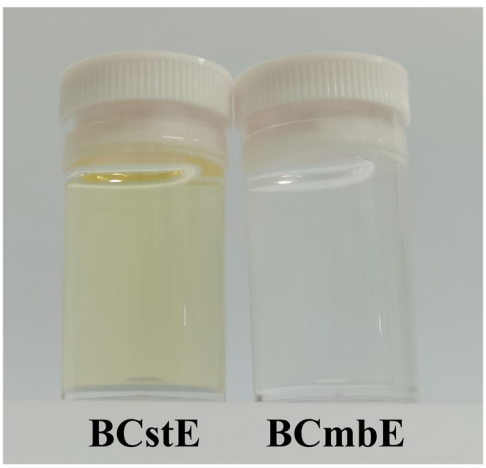

Fig. 1 | The picture of the bread crust and crumb extracts.

Table 3 | Fluorescent AGEs content in the BCst, BCmb, BCstE and BCmbE

Sample	Fluorescent AGEs content (AU)		
	Glycosylated collagen	Pentosidine	Pyrrole and imidazole derivatives
BCst	63770.0 ± 585.06^{a}	54670.0 ± 272.21^{a}	76880.0 ± 437.15^{a}
BCmb	9911.0 ± 79.96^{b}	13380.0 ± 68.74^{c}	17170.0 ± 99.97^{c}
BCstE	10374.0 ± 112.09^{b}	14524.0 ± 108.89^{b}	19212.0 ± 129.94^{b}
BCmbE	1788.2 ± 6.69^{c}	2802.0 ± 19.28^{d}	4231.6 ± 33.35^{d}

Reported values are the mean ± SD (n = 3). Values bearing different superscripts in the same column are significantly different (p < 0.05). AGEs: advanced glycation end products.

Antioxidant capacity

The antioxidant capacity of BCstE was significantly greater than that of BCmbE, which was measured by oxygen radical absorbance capacity (ORAC), ABTS (2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) and ferric reducing antioxidant power (FRAP) assays (Fig. 2). The difference in antioxidant capacity between bread crust and crumb is mainly due to the difference in antioxidative products at different stages of the Maillard reaction. Early Maillard reaction products seem to be not connected to antioxidant capacity[31]. The substances that contribute antioxidant capacity in bread are mainly products produced in the advanced and final stages of the Maillard reaction, such as melanoidins, reducing ketones and volatile heterocyclic compounds. Melanoidins were produced in the final stage of Maillard reaction, which has long been considered the major MRPs with high antioxidant properties[7]. This has been confirmed earlier in this paper that the Maillard reaction occurred more extensive in bread crust than in bread crumb, so the accumulation of Maillard reaction products with strong antioxidant power in the crust is higher than in the crumb. The previous studies have shown that pronylated lysine is a novel protein modification in bread crust melanoidins showing in vitro antioxidative and high amounts in the bread crust and low amounts in the crumb[12], further substantiating the experimental results of this study. As shown in Table 5, there was a significant positive correlation between the results of the antioxidant capacity of bread crust and crumb measured by ORAC, ABTS and FRAP assays, and a consistent trend was observed between the antioxidant capacity values of bread crust and crumb, so they were all suitable for the determination of antioxidant capacity of bread aqueous extracts. At the same time, there was a significant positive correlation among antioxidant capacity, browning intensity (yellowing index), the content of fluorescent AGEs and the content of total phenolic.

Spatial distribution of antioxidant capacity and fluorescent AGEs content in the cross-section of bread

As shown in Fig. 3, the antioxidant capacity and fluorescent AGEs content gradually increased from the inside (crumb) to the outside (crust) of baguettes. A positive correlation was observed between antioxidant capacity and fluorescent AGEs content. The analysis revealed that both the antioxidant capacity and the content of fluorescent AGEs were significantly reduced in parts J and K, located in the two end cross-sections, in contrast to parts 12, 13, and 14, which are situated in the middle cross-sections. The uneven distribution of antioxidant capacity and fluorescent AGEs content in the bread cross-section may be attributed to the use of water vapor to maintain oven temperature, which can lead to uneven temperature distribution. It's likely that temperatures reach their peak at the oven's bottom, causing the middle section of the dough to adhere to the baking pan

Table 4 | Extraction yield of fluorescent AGEs in the bread crust and crumb extracts

Sample	extraction yield (%)		
	Glycosylated collagen	Pentosidine	Pyrrole and imidazole derivatives
BCstE	16.27 ± 0.04^{b}	26.57 ± 0.07^{a}	24.99 ± 0.05^{a}
BCmbE	18.04 ± 0.08^{a}	20.94 ± 0.06^{b}	24.65 ± 0.07^{b}

Reported values are the mean ± SD (n = 3). Values bearing different superscripts in the same column are significantly different (p < 0.05).
AGEs advanced glycation end products.

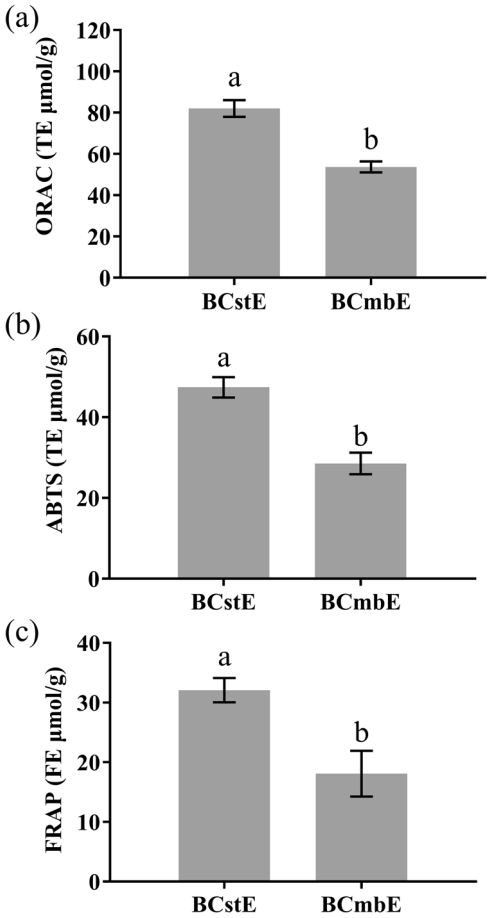

Fig. 2 | Antioxidant capacity of BCstE and BCmbE. Antioxidant capacity measured by ORAC (**a**), ABTS (**b**) and FRAP (**c**) assays. BCstE: baguette crust extracts, BCmbE: baguette crumb extracts, ORAC: oxygen radical absorbance capacity, ABTS: 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), FRAP: ferric reducing antioxidant power, TE: Trolox equivalent, FE: Fe^{2+} equivalent. The results were presented as mean ± SD based on triple measurements. Values followed by different letters indicate significant differences ($p \leq 0.05$) between different antioxidant capacity using one-way ANOVA followed by Tukey's test for post-hoc analysis. Error bars refer to standard deviation.

throughout the baking process. Meanwhile, the bread's end sections tend to deform and slightly elevate as the heat ascends.

Cytotoxicity of bread crust and crumb extracts

As determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method, the survival rate of macrophages in the presence of bread crust and crumb extracts at the tested concentrations was more than 80% and less than 110%, indicating that the samples had no acute toxic effect on macrophages, neither proliferation effect as shown in Fig. 4a. Therefore, the samples (crust and crumb) within the current concentration range are suitable for evaluating pro-inflammatory properties.

The disruption of cellular membrane integrity due to apoptosis or necrosis leads to the leakage of enzymes, including the stable lactate dehydrogenase (LDH), from the cytoplasm into the culture medium. Quantitative assessment of cytotoxicity can also be accomplished by measuring the activity of LDH released from cells with compromised plasma membranes into the culture medium[32]. As shown in Fig. 4b, bread crust and crumb extracts also exhibited no toxic effect on macrophages at the tested concentrations, which was consistent with the results determined by the MTT method. Surprisingly, the level of LDH in the cell supernatant was decreased in a concentration-dependent manner. Therefore, the LDH activity in the cell supernatant was further determined. The LDH activity in the supernatant was lower than that in the normal cell group and the LDH activity in the supernatant decreased with the increase of sample concentration after 24 h of co-incubation of bread crust and crumb extracts with cells (Fig. 4c). The results seem to show that bread crust and crumb extracts could mitigate natural cell damage under normal metabolism, exhibiting a protective effect on RAW264.7 macrophages, possibly by changing the permeability of cell membrane and leading to a decrease in lactate dehydrogenase activity in the cell supernatant. The observed experimental phenomenon may be related to antioxidant capacity[33,34].

Cellular antioxidant activity (CAA) of bread crust and crumb extracts

In Fig. 5a, the intracellular antioxidant activity of both samples exhibited a concentration-dependent trend. Specifically, the intracellular antioxidant activity of bread crust extracts in RAW264.7 macrophages was significantly higher than that of bread crumb extracts ($p < 0.05$), which was consistent with the results measured by the three chemical antioxidant assays (ORAC/ABTS/FRAP). The antioxidant activity of bread crust and crumb extracts was converted to an equivalent concentration of quercetin based on its CAA values, and the quercetin equivalents were determined to be 8.83 ± 0.52 μmol/g for BCstE and 2.21 ± 0.94 μmol/g for BCmbE, respectively. Previous studies have employed various methods to compare the disparity in antioxidant capacity between bread crust and bread crumb, indicating that the crust exhibits higher antioxidant activity compared to the crumb, which aligns with the trend observed in the experimental outcomes of this study[12,31,35]. Notably, this study introduces the first application of CAA method to assess the intracellular antioxidant activity of baguette crust and crumb, providing a more accurate reflection of the bread's biological activity.

Cell membrane potential and mitochondrial superoxide of RAW264.7 macrophages

Macrophages, key players in the immune system, exhibit cell membrane potential and mitochondrial superoxide levels as critical indicators of their viability and functional state, reflecting their health and physiological roles. As depicted in Fig. 5b–f, incubation of RAW264.7 macrophages with either BCstE or BCmbE did not affect the green fluorescence emitted by DiBAC4(3) on RAW264.7 macrophages, indicating neither BCstE nor BCmbE affect the cell membrane potential (V_{mem}) of normal RAW264.7 macrophages, even at a high concentration (BCstE and BCmbE diluted 5 times, respectively). In the presence of AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride), the fluorescence of DiBAC4(3) in RAW264.7 macrophages was nearly eliminated (Fig. 5b), indicating the rising V_{mem} and hyperpolarization of cytosolic membrane caused by AAPH-induced peroxyl radicals. Membrane hyperpolarization is known to be paralleled with the activation of macrophage functions, i.e., NO production[36]. In contrast, both BCstE and BCmbE completely or partially restored the V_{mem} in AAPH insulted cells in a dose-dependent manner (Fig. 5c–f), counteracted the membrane hyperpolarization caused by the extracellular peroxyl radicals. The BCstE mitigated the peroxyl radical-induced cell membrane hyperpolarization by 91% at a high concentration (sample diluted 5 times), showing higher activities than the BCmbE. Therefore, BCstE and BCmbE did not affect cytosolic membrane of normal macrophages, but could significantly protect cytosolic membrane from AAPH-induced oxidative damage.

Regardless of the influence on the intracellular or extracellular stimulation of macrophages, the mitochondrial reactive oxygen species (ROS) levels serve as a sensitive indicator. The peroxyl radical-induced oxidative stresses suppressed the mitochondrial oxygen respiration and caused a decrease in mitochondrial ROS[19]. It is shown in Fig. 5b, g, incubation of RAW264.7 macrophages with neither BCstE nor BCmbE affect the fluorescence of ROS-sensitive dye Mito-SOX Red (also named Mito-HE) in RAW264.7 macrophages. However, in the presence of AAPH, a notable reduction in the fluorescence of Mito-HE was observed, suggesting a decrease in ROS levels within the mitochondria resulting from the

Table 5 | Correlation analysis of indicators in the bread crust and crumb extracts

Index	Antioxidant capacity									Fluorescent AGEs	
	ORAC	ABTS	FRAP	Protein	Carbohydrate	TG	TPC	YI	Glycosylated collagen	Pentosidine	Pyrrole and imidazole derivatives
ORAC	1.00										
ABTS	0.98**	1.00									
FRAP	0.85*	0.90*	1.00								
Protein	0.92**	0.87*	0.59	1.00							
Carbohydrate	−0.24	−0.43	−0.31	−0.24	1.00						
TG	−0.61	−0.68	−0.821*	−0.31	0.45	1.00					
TPC	0.94**	0.99**	0.92**	0.82*	−0.46	−0.67	1.00				
YI	0.99**	0.99**	0.87*	0.90*	−0.32	−0.60	0.97**	1.00			
Glycosylated collagen	0.99**	0.99**	0.91*	0.87*	−0.29	−0.67	0.97**	0.99**	1.00		
Pentosidine	0.99**	0.99**	0.89*	0.89*	−0.32	−0.66	0.97**	0.99**	1.00**	1.00	
Pyrrole and imidazole derivatives	0.99**	0.99**	0.90*	0.88*	−0.32	−0.66	0.98**	0.99**	1.00**	1.00**	1.00

ORAC oxygen radical absorbance capacity, *ABTS* 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), *FRAP* ferric reducing antioxidant power, *TG* triglyceride, *TPC* total phenolic contents, *YI* yellowing index, *AGEs* advanced glycation end products.
*: $p < 0.05$;
**: $p < 0.01$.

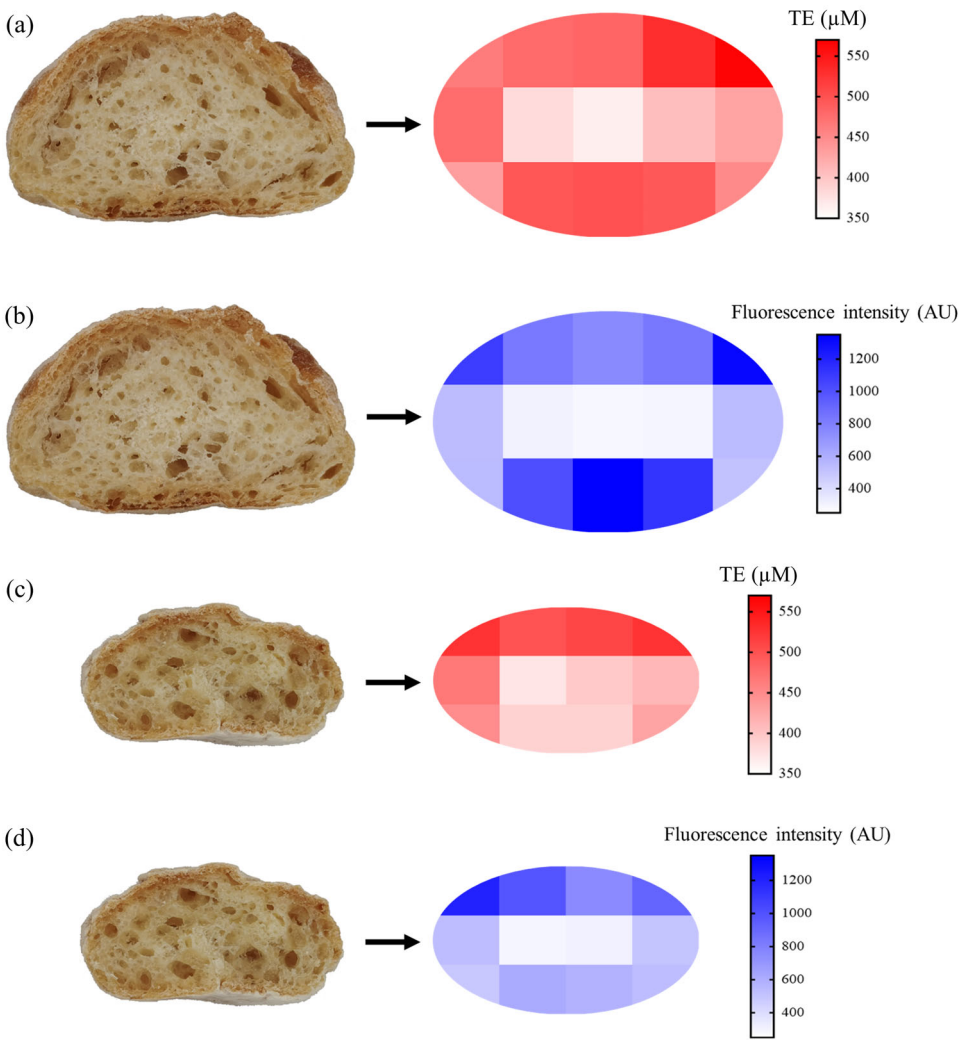

Fig. 3 | Antioxidant capacity and fluorescent AGEs heat map of the cross-section of baguette. **a** Antioxidant capacity heat map of the middle part's cross-section (ORAC assay); (**b**) fluorescent AGEs heat map of the middle part's cross-section; (**c**) antioxidant capacity heat map of cross-section at both ends (ORAC assay); (**d**) fluorescent AGEs heat map of cross-section at both ends. AGEs advanced glycation end products, ORAC oxygen radical absorbance capacity, TE Trolox equivalent.

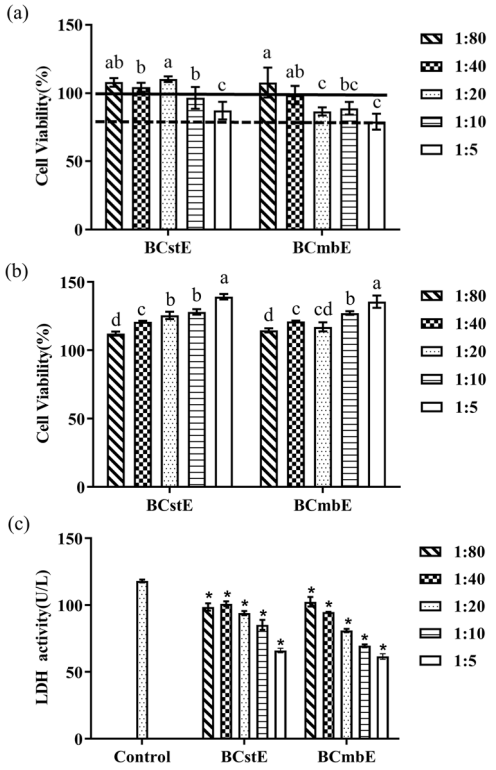

Fig. 4 | Cytotoxicity of bread crust and crumb extracts. a Cytotoxicity of bread crust and crumb extracts in RAW264.7 macrophages was determined by MTT method (The solid line in the figure indicates that the cell survival rate is 100%, and the dotted line indicates that the cell survival rate is 80%); (**b**) LDH toxic effects of bread crust and crumb extracts in RAW264.7 macrophages; (**c**) the activity of LDH in the cell supernatant after the sample was co-cultured with RAW264.7 macrophages for 24 h. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, LDH lactate dehydrogenase. The results were presented as mean ± SD based on triple measurements. Different letters indicate significant differences ($p \le 0.05$) using one-way ANOVA followed by Tukey's test for post-hoc analysis, $^{*}p < 0.05$. Error bars refer to standard deviation.

impairment of oxygen respiration in the organelle induced by AAPH radicals. Incubation of RAW264.7 macrophages together with AAPH and either BCstE or BCmbE completely or partially counteracted this suppression on mitochondrial oxygen respiration in a dose-dependent manner. The BCstE mitigated the suppression on the oxygen respiration in mitochondria by up to 100% at a high concentration (BCstE diluted 5 times), whereas the BCmbE mitigated this suppression by up to 79% at a high concentration (BCmbE diluted 5 times). Hence, it can be concluded that BCstE exhibited higher activities compared to BCmbE.

Inflammatory factors in supernatant of RAW264.7 macrophages

As shown in Table 6, within a certain concentration range, neither BCstE nor BCmbE induced the production of IL-1β and IL-6, and the BCmbE did not induce the production of TNF-α. However, the BCstE was found to induce TNF-α level in RAW264.7 macrophages when diluted 5–40 times, indicating that the BCstE causes a slight inflammatory response in these macrophages. However, it neither promotes nor inhibits the production of IL-1β and IL-6 under standard conditions.

Two assumptions are proposed based on the experimental results: (1) a large number of AGEs in the BCstE can disrupt the balance of oxidative stress in cells through the receptors for advanced glycation end products (RAGEs), leading to an increase in the secretion level of pro-inflammatory cytokine TNF-α[37]. However, the fluorescent AGEs content in BCmbE is significantly lower than that in BCstE, which did not translate into an obvious effect on the release of TNF-α by macrophages. Although the previous experiments confirmed that BCstE exhibited intracellular antioxidant activity, it was observed over a short period of time, and its prevalence in the medium may not be sufficient to counteract the oxidative stress effects of AGEs present in this extract on RAW264.7 macrophages. Moreover, it is also known that the fluorescent determination of AGES does not consider the non-fluorescent, including both non-cross-linking and cross-linking AGEs which can have a distinct effect. (2) The presence of gluten protein in the BCstE induces the production of TNF-α, leading to systemic inflammation. The possible reason is that gluten protein triggers adaptive immune responses mediated by $CD4^{+}$Th1 cells and innate immune responses mediated by intraepithelial lymphocytes, which damage intestinal epithelial inflammatory cells[38]. Crust extract does indeed show a higher content of protein, but it does not specify whether this attribute is related to gluten protein. Both explanations could potentially work in synergy, resulting in the observed effects.

Inflammatory factors in supernatant of RAW264.7 macrophages co-incubated with lipopolysaccharide

To explore whether bread water extract can inhibit the production of IL-1β and IL-6 in macrophages under stress, an experiment was conducted to observe the effect of samples on the inflammatory factors released by cells stimulated by lipopolysaccharide (LPS), a known stimulant of immune responses.

According to Table 7, RAW264.7 macrophages exhibited an inflammatory response with the cells releasing 26.11 pg/mL IL-1β and 39.53 pg/mL IL-6 when stimulated by LPS. LPS can induce RAW264.7 macrophages to generate free radicals, leading to oxidative damage to cells and consequently promoting the expression and release of inflammatory factors. Both BCstE and BCmbE exhibited varying degrees of inhibitory effects on the production of IL-1β and IL-6 by RAW264.7 macrophages. This can be attributed to their capacity to express intracellular antioxidant activity, which mitigates the inflammatory response caused by ROS, thereby reducing the release of IL-1β and IL-6.

However, as the concentration decreased, the inhibitory effects of BCstE and BCmbE on the production of IL-1β and IL-6 increased, with BCstE showing a less pronounced inhibition compared to BCmbE. The underlying reason for the crumb extract's significant inhibitory effect remains unclear, sparking curiosity among researchers.

Methods

Materials

High gluten flour (Weifang Fengzheng Flour Co., Ltd., Shandong, China). Instant dry yeast and bread improver (Angel Yeast Co., Ltd., Hubei, China). 96-well plate with clear/black well, T-75 and T-25 cell culture flask were obtained from Corning Incorporated (New York, USA). Phosphate buffer saline (PBS), Hanks' balanced salt solution (HBSS), fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), penicillin-streptomycin, 0.25% Trypsin-EDTA were purchased from GIBCO (Grand Island, NY, USA). Triglyceride (TG) kit, Lactate dehydrogenase (LDH) assay kit and LDH were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). LDH cytotoxicity assay kit, human IFN-α/IFN-β/IFN-γ ELISA kit, bicinchoninic acid (BCA) kit, total antioxidant capacity (T-AOC) assay kit with ABTS method and T-AOC assay kit with FRAP method were purchased from Beyotime Biotechnology (Shanghai, China). MTT, 2',7'-dichlorofluorescein diacetate (DCFH-DA, ≥97% purity), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), dimethyl sulfoxide (DMSO), Trolox (≥98% purity by HPLC), quercetin dihydrate (≥98% purity by HPLC), Nile Red, DiBAC4(3), Mito-SOX Red were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). High purity deionized water obtained using a Millipore Milli-Q Academic deionizer system (resistivity 18.2 MΩ cm, Bedford, MA, USA). All other chemicals and solvents used were of AR grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

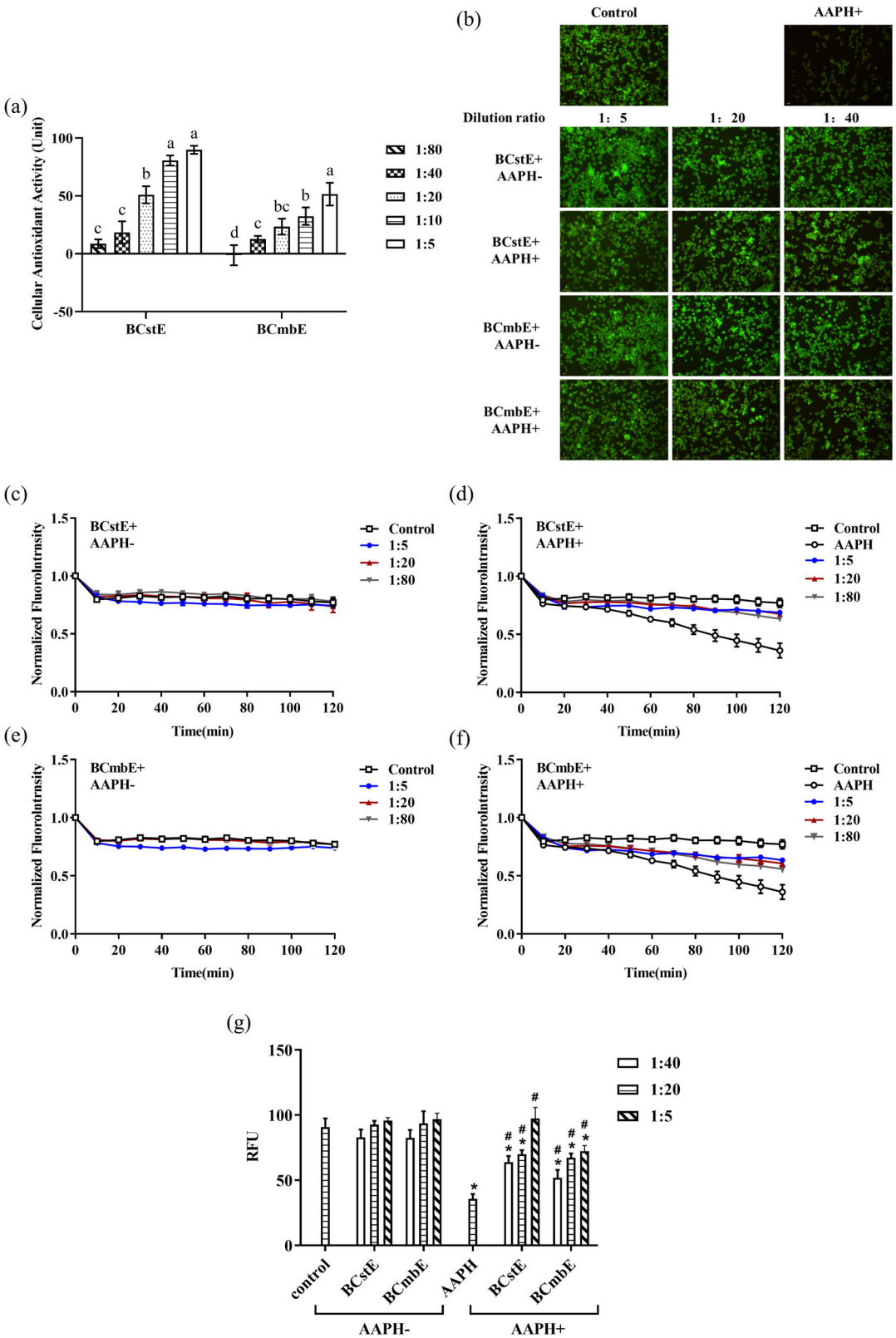

Fig. 5 | Cellular antioxidant activity of BCstE and BCmbE and effects of BCstE and BCmbE on cell membrane potential and mitochondrial superoxide level in RAW264.7 macrophages. a Cellular antioxidant activity of BCstE and BCmbE in RAW264.7 macrophages; (**b**) effects of BCstE and BCmbE on the membrane potential of cells plasma (indicated with green fluorescence of DiBAC4(3)) and mitochondrial superoxide (indicated with red fluorescence of MitoSOX) of RAW264.7 macrophages; (**c**–**f**) effects of BCstE and BCmbE on the membrane potential of RAW264.7 macrophages; (**g**) effects of BCstE and BCmbE on the mitochondrial superoxide level of RAW264.7 macrophages. BCstE baguette crust extracts, BCmbE baguette crumb extracts, AAPH 2,2′-azobis (2-amidinopropane) dihydrochloride. The results were presented as mean ± SD based on triple measurements. Different letters indicate significant differences using one-way ANOVA followed by Tukey's test for post-hoc analysis, *: $p < 0.05$ vs Control, #: $p < 0.05$ vs AAPH. Error bars refer to standard deviation.

Table 6 | Inflammatory factors in the supernatant of RAW264.7 macrophages after incubating with BCstE or BCmbE

Sample	TNF-α/(pg/mL)	IL-1β/(pg/mL)	IL-6/(pg/mL)
Control	ND	ND	ND
BCstE(1:5)	3.02 ± 1.56	ND	ND
BCstE(1:40)	3.20 ± 2.07	ND	ND
BCmbE(1:5)	ND	ND	ND
BCmbE(1:40)	ND	ND	ND

"ND" indicates that not detected.

Table 7 | Inflammatory factors in the cell supernatant after incubating of RAW264.7 macrophages together with LPS and BCstE or BCmbE

Sample	IL-1β/(pg/mL)	IL-6/(pg/mL)
LPS	26.11 ± 13.84^{a}	39.53 ± 3.55^{a}
BCstE (1:5) + LPS	18.05 ± 2.42^{b}	16.18 ± 2.29^{b}
BCstE (1:40) + LPS	7.80 ± 5.72^{c}	4.53 ± 7.86^{c}
BCmbE (1:5) + LPS	2.97 ± 4.01^{c}	ND
BCmbE (1:40) + LPS	1.75 ± 3.47^{c}	ND

LPS lipopolysaccharide.
"ND" indicates that not detected. Reported values are the mean ± SD ($n = 3$). Values bearing different superscripts in the same column are significantly different ($p < 0.05$).

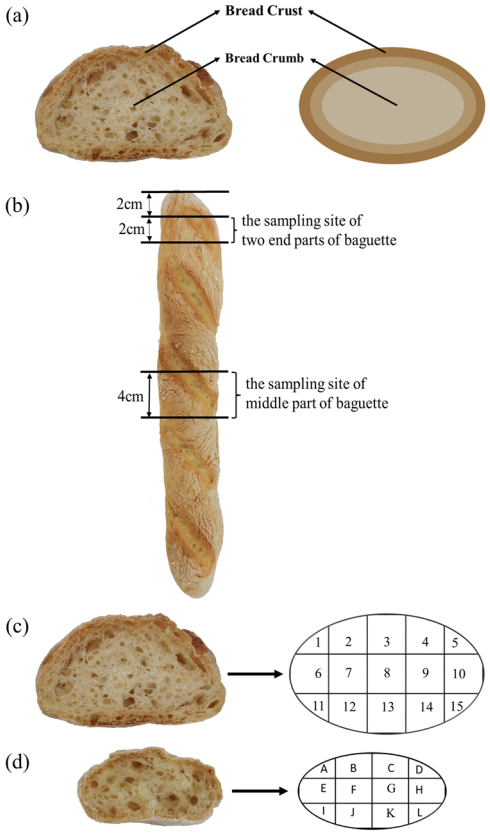

Fig. 6 | Schematic diagram of the sampling sites. a The sampling sites of crust and crumb sample of baguette cross-section; (**b**) the sampling sites of the middle and two end parts of baguette; (**c**) the sampling sites of the middle cross-section of baguette (different numbers represent different sampling sites); (**d**) the sampling sites of two end cross-sections of baguette (different letters represent different sampling sites).

Samples preparation

For baguette making, the dough formula was 450 g flour, 4 g dry yeast, 2.25 g bread improver, 4.5 g salt and 342 g water. The ingredients were mixed in a mixer (HK-TU10L, Shanghai Baker 's Kingdom Co., Ltd., Shanghai, China) for 10 min at medium speed. After a 3.5 h fermentation period, the dough was gently sheeted into its final form and then placed into a steam oven (SM2-522, Sinmag Machinery Co., Ltd., Jiangsu, China) preheated to 230 °C for 30 min. Subsequent to baking process, the baguette was left to stand for 24 h at room temperature, and then kept frozen (at −20 °C) until analysis[1,3].

As shown in Fig. 6a, b, 2 cm parts from 2 cm away from the end and 4 cm parts in the midpoint of the entire baguette were cut, and the preliminary baguette crust and crumb samples were separated, respectively. After ventilation and drying at 25 °C, the preliminary baguette crust and crumb were dried and then ground in a mill and screened through a 0.15 mm sieve to obtain powdered baguette crust (BCst) and baguette crumb (BCmb), stored at −20 °C.

The baguette crust extracts (BCstE) and baguette crumb extracts (BCmbE) were prepared optimally by following the recommended material-to-water ratio and vortex oscillation time, according to a previous study[39]. Briefly, 3 g powdered baguette crust or crumb was mixed with 30 mL deionized water, respectively, and vortexed for 30 min and placed for 30 min at room temperature. The supernatant infusion was obtained by centrifuging the mixture for 15 min at 5813 g and 25 °C. Subsequently, the resulting BCstE and BCmbE were carefully collected and stored at 4 °C for preservation. Each extract was then transferred to a pre-weighed glass dish with a 9 cm diameter and subjected to drying at 105 °C for 3 h to determine the dry weight.

Physicochemical characterization

Protein content was determined by using a bicinchoninic acid (BCA) kit with bovine serum albumin (BSA) as the standard sample. The content of carbohydrate concentration was determined by the anthrone-sulfuric acid assay, using glucose as the standard[40]. Triglyceride (TG) content was determined by TG kit with GPO-PAP assay. Total phenolic contents (TPC) was determined by using the Folin-Ciocalteau reagent[41]. The turbidity of extraction was determined with an UV/VIS spectrophotometer (UV-5100, HITACHI, Tokyo, Japan) at 600 nm[42].

Evaluation of browning intensity

The color of baguette samples was determined using a Chroma Meter (CR-400, Konica Minolta, Tokyo, Japan) according to the CIELab scale. The CIELab color scale, developed by the International Commission on Illumination, is a color space that describes all the colors visible to the human eye. Color was expressed in L^*, a^*, b^* Hunter scale parameters: L^* or Luminance (0 to 100), a^* or coloring from green to red (-120 to 120), b^* or coloring from blue to yellow (-120 to 120). The browning intensity was indicated by the yellowing index (YI)[43], which was calculated using the following Eq. (1).

$$YI = 142.86b^*/L^* \quad (1)$$

Measurement of fluorescent AGEs

Measurement of fluorescent AGEs was using fluorescence assays[44,45]. 3 g powdered baguette crust or crumb was mixed with 30 mL Tris-HCl buffer (pH = 7.5) containing 36 mg streptomycin proteinase E, 150 mg sodium dodecyl sulfate and 44.1 mg $CaCl_2$, and then was digested by shaking in a shaker at 30 °C for 36 h. The dispersions were centrifuged at 4500 × *g* for 10 min at 4 °C, and the supernatants were diluted with ultrapure water for further analysis. The solution was measured by fluorescence spectrophotometry at excitation/emission wavelengths of 370/440 nm (glycosylated collagen), 330/420 nm (pyrrole and imidazole derivatives) and 335/385 nm (pentosidine), respectively. The parameters of the fluorescence

spectrophotometer were set as: the voltage of the photomultiplier tube was 700 V, the response time was 0.5 s, the slit width was 5.0 nm on the excitation side and 5.0 nm on the emission side, and the scanning mode was time scanning for 20 s.

Determination of antioxidant activities

Antioxidant activities of BCstE and BCmbE were assessed with by the following methods: (i) oxygen radical absorbance capacity (ORAC) assay, (ii) total antioxidant capacity (T-AOC) assay kit with ABTS method and (iii) T-AOC assay kit with FRAP method.

ORAC assay was carried out according to a previous method with slight modifications[18]. 25 μL of samples diluted by phosphate buffer (75 mM, pH = 7.4) and Trolox standard solutions (3.125, 6.25, 12.5, 25, 50, 100 μM) were added to clear 96-well plates in triplicate, followed by 150 μL sodium fluorescein (8.61×10^{-5} mM) to each well. After the incubation at 37 °C for 15 min, AAPH (152.66 mM, 25 μL) were added to initiate the oxidation reaction, while the phosphate buffer was set as blank. The fluorescence intensity was recorded every 2 min for 180 min at Ex485 nm and Em520 nm, respectively (FlexStation 3 Plate Reader, Molecular Devices, Sunnyvale, CA, USA). The data were expressed as TE μmol/g of samples according to the Eq. (2) as below:

$$\text{ORAC value (TE}\,\mu\text{mol/g)} = \text{Trolox equivalent/Dry weight of sample} \quad (2)$$

Spatial distributions of antioxidant capacity and fluorescent AGEs in baguette cross-sections

The spatial distributions of antioxidant capacity and fluorescent AGEs were achieved by grid sampling in baguette cross-sections. As shown in Fig. 6b, the middle and two end parts of baguette were carefully separated by a knife. The sampling schematic diagram of the transverse and longitudinal isometric sections of baguette as shown in Fig. 6c, d. The middle cross-section was divided into 5 transverse isometric parts and 3 longitudinal isometric parts, with different sampling sites labeled by numbers 1-15. The two end cross-sections were divided into 4 transverse isometric parts and 3 longitudinal isometric parts, with different sampling sites labeled by letters A-L. Then the antioxidant capacity and fluorescent AGEs content of samples were determined, respectively. The fluorescent AGEs were determined by fluorescence assay[44,45] and determination of antioxidant capacity was using ORAC assay[18].

Cell culture

RAW 264.7 macrophages (Shanghai Institute of Life Sciences, Chinese Academy of Science, China) were grown at standard cell culture conditions (5% CO_2, 37 °C) in a T-75 cell culture flask with Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells between passages 10 and 20 were used in this study.

Determination of cytotoxicity

Cytotoxicity of BCstE and BCmbE was determined by MTT assay[46]. RAW 264.7 macrophages were seeded in 96-well plates. After incubation for 24 h, the cell culture medium was removed and washed once with HBSS. Samples were diluted to appropriate gradient concentrations with complete DMEM. Adding 200 μL of the diluted samples to 96-well cell culture plates and setting control wells and zeroing wells. Following another 24 h incubation, the cell morphology was observed under an inverted microscope (DMI3000 B, Leica, Wetzlar, Germany). 20 μL of solution (5 mg/mL) was added to the wells, followed by a further 3 h incubation in the dark. The cell culture medium with MTT was removed and washed twice with HBSS. 200 μL of dimethyl sulfoxide (DMSO) was added to the wells, and the plate was shaken on an oscillator for 10 min. The absorbance at 570 nm was recorded using a FlexStation 3 Plate Reader for measuring cell viability.

Cytotoxicity of BCstE and BCmbE was also determined by LDH assay. RAW 264.7 macrophages were initially seeded in 96-well plates. Following a 24 h incubation period, the cell culture supernatant was collected and subjected to detection using an LDH cytotoxicity detection kit. The procedure strictly adhered to the instructions provided with the kit to ensure accuracy and reliability in the cytotoxicity assessment.

CAA assay

The CAA assay was carried out following the method of reference[47]. RAW 264.7 macrophages were plated (6.0×10^4 cell/well) in 200 μL cell culture media/well in 96-well black. After incubation for 24 h, the cell culture medium was removed and washed twice with HBSS. Samples and standard substance (Quercetin) were diluted to appropriate gradient concentrations with complete DMEM. The diluted samples or standard substance (100 μL) and DCFH-DA (50 μM, 100 μL) were added to each well in triplicate wells, and control wells and blank wells were set up. After 1 h incubation at 37 °C, RAW 264.7 macrophages were washed once by HBSS. Then 100 μL of AAPH (600 μM) was added to the wells and the plate was immediately placed in a FlexStation 3 Plate Reader. Real-time fluorescence was read initially and every five minutes after for 1 h with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Control wells were treated with DCFH-DA and AAPH without antioxidants, while blank wells were treated with DCFH-DA without AAPH and antioxidants. area under the fluorescence curve (AUC) was calculated with GraphPad Prism 5.0. Antioxidant activity was expressed as μmol/g quercetin equivalents using the linear regression value obtained from the quercetin calibration curve ($y = 0.055x + 0.3326$, $R^2 = 0.9907$). The result was calculated using the following Eq. (3).

$$CAA_{unit} = \left(1 - \frac{AUC_{sample}}{AUC_{control}}\right) \times 100 \quad (3)$$

Cell membrane potential and mitochondrial superoxide determination

RAW 264.7 macrophages were seeded in 96-well black plates at 2×10^4 cells/well. After incubation for 24 h, the wells were washed twice with HBSS, and then Mito-SOX Red (200 μL, 3 μM in HBSS) was added to each well which at 37 °C for 10 min. 100 μL of 5 μM DiBAC4 (3) was added to each well and incubated for 15 min. Baguette samples (50 μL/well) were added at three ratios with HBSS as the negative control. AAPH was added into each well as the positive control group to reach a final concentration of 6.4 mM. Put the 96-well plate into FlexStation 3 Plate Reader and read the data every 10 min for 120 min at 37 °C. The excitation and emission wavelength for Mito-SOX Red was set at Ex510 nm and Em580 nm, and that of DiBAC4(3) was set at Ex493 nm and Em516 nm. The fluorescent micrographs were captured immediately following the fluorometric measurement using an inverted microscope (DMI3000 B, Leica, Wetzlar, Germany). Ex515-560 nm and Em590 nm were used for Mito-SOX Red, Ex450-490 nm and Em516 nm were used for DiBAC4(3)[19]. Duplicates of the above tests were performed to calculate the membrane potential and mitochondrial superoxide levels.

Inflammatory cytokines determination

RAW 264.7 macrophages were seeded in 12-well plates. After incubation for 24 h, the cell culture supernatant was removed. 1 mL of BCstE diluted with DMEM (BCstE/DMEM = 1:5, BCstE/DMEM = 1:40) was added, setting up a control group. BCmbE was treated in the same manner. After co-culture for 24 h, the cell supernatant was collected and the levels of TNF-α, IL-1β and IL-6 were detected by ELISA kits. To determine the effect of the samples on the inflammatory factors released by cells stimulated by lipopolysaccharide (LPS), the cells were seeded in 12-well plates and cultured for 24 h. After removing the cell culture supernatant, 500 μL of 40 μg/mL LPS diluted with DMEM was added, and the cells were cultured for 1 h in the incubator. Adding 500 μL of BCstE and BCmbE diluted 5-fold/40-fold with DMEM respectively and setting a positive control group (500 μL each

DMEM and LPS, the specific operation steps were consistent with the sample group). After co-incubated for 24 h, the supernatant of cells was collected and the levels of IL-1β and IL-6 were determined by ELISA kits.

Statistical analysis

All experiments were performed at least three times to ensure reproducibility and reliability. Statistical analysis was conducted using GraphPad Prism version 5.0 and SPSS version 27.0. Significant differences were determined by using one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered statistically significant.

Data availability

The authors declare that the data supporting the findings of this study are available within the article.

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Author contributions

J.Z.: Investigation, Data curation, Methodology, Writing original draft. Z.Y.: Funding acquisition, Methodology, Validation, Writing—review & editing. F.H.: Data collection and analysis. S.L.: Data collection and analysis. F.M.C.-G.: Writing—review & editing. L.K.: Conceptualization, Supervision, Writing—review & editing. H.G.: Funding acquisition, Supervision, Writing—review. P.W.: Conceptualization, Supervision, Writing—review & editing.

Competing interests

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. LK is Associate Editor of npj Science of Food. LK was not involved in the journal's review of, or decisions related to, this manuscript.

Additional information

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