The impact of high hydrostatic pressure treatment on anthocyanins, colour, microorganisms, and enzyme activity of mulberry (*Morus nigra*) juice

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Abstract

The effects of high hydrostatic pressure (HHP) treatments at 200, 400, and 600 MPa, for 10, 20, and 30 min on residual activities of polyphenol oxidase (PPO) and peroxidase (POD), colour properties, and microbial populations of total viable count, yeasts, and moulds of mulberry juice were determined with reference to untreated mulberry juice. Lower pressure levels were found to significantly inactivate PPO activity than higher pressure level. A strong positive relationship (R = 0.947) existed between PPO activity and increases in pressure and time. Also, a moderately positive relationship existed (R = 0.358) between POD activity and increases in pressure and time, but the relationship was not statistically significant. Anthocyanin content was better retained at lower pressure levels than at higher levels, with sample treated at 200 MPa/10 min retaining 96.50 ± 0.74%. An inverse relationship was observed between anthocyanin retained and residual PPO (R = -0.425) and POD (-0.075) activities, but the relationships were not statistically significant. Total viable count as well as yeast and mould count of all HHP treated mulberry juice samples were < 10 CFU/mL with $\log_{10}$ 4.86, 4.45, and 4.83 CFU/mL reductions, respectively. Also, colour parameters of brightness, hue angle, chroma, and total colour difference were differently affected. Treatment at 200 MPa/10 min gave a better inactivation of PPO and POD, better retention of anthocyanin, and preserved the colour of the mulberry juice to almost the same extent as the control, making it the best treatment to be adopted.

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Introduction

The use of high hydrostatic pressure (HHP) is gaining industrial recognition and application as many products are now being processed using the technology due to the comparative advantage it has over other conventional processing technologies such as thermal (Cheftel, 1995; Hayashi, 1996; Patras *et al.,* 2009). HHP offers various technological advantages such as the attainment of an almost immediate isostatic pressure transmission to the product independent of package size, shape, and food composition, resulting in highly homogeneous products (Patterson *et al.,* 1996; Rastogi *et al.,* 2007). HHP is also known to better preserve the nutritional, health-promoting, as well as sensory properties of food over other known methods of food preservation (Zabetakis *et al.,* 2000; Patras *et al.,* 2009). Conventional methods, particularly thermal processing, are known to have negative effects on the nutritional (water-soluble vitamins) as well as other health promoting phytochemicals such as anthocyanins,

antioxidant activity, and colour (Fazaeli *et al.,* 2013; Chong *et al.,* 2013; Engmann *et al.,* 2014). HPP is capable of interrupting large molecules such as proteins or microbial cell structures, enzymes, lipids, and cell membranes, leaving small molecules such as vitamins and flavour components unaffected (Linton and Patterson, 2000). The effect of HHP on many food materials and their components (anthocyanins, total antioxidant activity, phenolic, ascorbic acid, and colour) have been investigated and reported by many (Patras *et al.,* 2009; Engmann *et al.,* 2013). However, little work has been done on the effect of HHP on mulberry juice.

Mulberry fruits are a rich source of anthocyanins (Yang and Tsai, 1994). Anthocyanins are a group of water-soluble pigments with powerful antioxidant, antitumour, and antiradical properties which are known to have therapeutic as well as preventive properties (Konczak and Zhang, 2004; Umar Lule and Xia, 2005; Nichenametla *et al.,* 2006). It is therefore imperative that processing technologies adopted for processing mulberry juice should help to

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promote health by preserving its anthocyanins, nutrients, and other phytochemicals, while inactivating enzymes and microorganisms.

It is known that polyphenol oxidase (PPO) and peroxidase (POD) activities of different fruit juices are differently affected by HHP (Cheftel, 1995; Cano *et al.,* 1997). Therefore, it is important to investigate the effect of HHP on mulberry juice enzymes at different pressure and time levels. This is necessary as there is a correlation between residual enzyme activity and rate of degradation of colour in fruit juices as a result of destruction of anthocyanins (Zapata *et al.,* 1995).

The objective of the present work was therefore to investigate the effect of HHP treatment regimes on anthocyanin retained, inactivation of microorganisms, PPO and POD activities, and colour parameters of brightness, hue angle, chroma and total colour difference of mulberry juice.

Materials and methods

Sample source and preparation

Mulberry fruits (*Morus nigra* var. Zhen Jiang No. 1) were purchased from a farm along the Yangtze River Zhenjiang, P.R. China. The fruits were washed and frozen at -8°C until used. Before use, the fruits were thawed at 5°C and then macerated in a household blender. The puree obtained was centrifuged (Beckman Coulter Avanti J-25 centrifuge, Beckman Instruments, Inc., California) at 5,000 rpm for 20 min at 4°C. The supernatant was filtered through a cheese cloth and immediately packaged.

High hydrostatic pressure treatment

Packaged samples were put in a second polythene pouch and sealed before the HHP treatment. The HHP treatment was carried out at 8 ± 2°C for all the pressure and time regimes using the "Intelligent super high pressure food processing device" (Jiangsu, P.R. China). It had a height of 440 mm, external diameter of 290 mm, internal diameter of 150 mm, and volume of 6.5 L. The pressurising liquid used was di-octyl sebacate. The system had a pressure vessel, hydraulic intensifier, a pump, and a control panel. The come-up time for attaining the maximum pressure of 600 MPa was 105 s. Three levels of pressure and three levels of time were employed in treating mulberry juice samples. The various combinations are shown in Table 1.

Anthocyanin determination

The pH differential method (Lee *et al.,* 2005) was employed to determine the total monomeric

Table 1. Factor combinations for high pressure treatment of mulberry juice samples.

Sample	Pressure (MPa)	Time (min)
A	200	10
B	200	20
C	200	30
D	400	10
E	400	20
F	400	30
G	600	10
H	600	20
I	600	30

anthocyanin content of samples. Before analysis, juice samples were centrifuged (TLG-20M, Hunan Changsha Xiang Yi Centrifuge Instrument Co., Ltd) at 10,000 rpm for 20 min at 4°C. Absorbance was read at 520 and 700 nm using distilled water as blank and anthocyanin content calculated using Equation 1:

$$\text{Anthocyanin (mg/L)} = (A \times MW \times DF \times 10^3) / (\varepsilon \times L) \quad \text{(Eq. 1)}$$

where, A (absorbance) = $(A_{520nm} - A_{700nm})$pH 1.0 - $(A_{520nm} - A_{700nm})$pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin 3-glucoside, being the major anthocyanin in mulberry juice (Singhal *et al.,* 2010; Engmann *et al.,* 2013); DF (dilution factor) = 100; 10^3 is the conversion factor from gram to milligram; ε (molar absorbance) = 26,900 Lmol^{-1}cm^{-1} for cyanidin 3-glucoside; and L = light path length (1.0 cm).

Enzyme extraction

The method adopted for analysing PPO and POD activities was as described by Garcia-Palazon *et al.* (2004) with some modifications. Sodium phosphate buffer (SPB) of 0.2 M with pH 6.5, 4% (w/v) polyvinylpyrrolidone (PVP), 1% triton X-100, and 1 M sodium chloride, made up the extraction solution. Equal volumes of the extraction solution and mulberry juice were mixed and centrifuged (TLG-20M, Hunan Changsha Xiang Yi Centrifuge Instrument Co., Ltd) at 10,000 *g* for 20 min at 4°C. The supernatant was filtered using a Whatman No. 1 filter paper (Whatman Intl. Ltd., Maidstone, England) to obtain the crude enzyme extract.

PPO activity measurement

To measure PPO activity, the reaction mixture, prepared at 4°C, comprised of 100 μL enzyme extract and 3.0 mL of 0.07 M catechol in

0.05 M SPB (pH 6.5). PPO activity was checked by measuring the absorbance of the assay, due to the formation of benzoquinone, at 420 nm for 10 min using a UV spectrophotometer (UV-1600, Beijing Rayleigh Analytical Instrument Co., Ltd). In the blank, the enzyme extract was substituted with 0.2 M SPB (pH 6.5). The activity of the enzyme was computed as the change of absorbance/min/0.1 mL of mulberry juice extract.

POD activity measurement

To measure POD activity, 100 μL of crude enzyme extract, 4.0 mL of 0.05 M SPB (pH 6.5), 100 μL of 1% p-phenylenediamine in 0.05 M SPB (pH 6.5), and 100 μL of 1.5% hydrogen peroxide were mixed. The blank was prepared in similar manner with crude enzyme extract being replaced with 0.2 M SPB (pH 6.5). The assay was prepared at 4°C and absorbance measured at 485 nm for 5 min using a UV spectrophotometer (UV-1600, Beijing Rayleigh Analytical Instrument Co., Ltd). The activity of POD was expressed as the change of absorbance/min/0.1 mL of mulberry juice extract. The residual activity (RA) of the enzymes (PPO and POD) was estimated using Equation 2:

RA = (enzyme specific activity after treatment / enzyme specific activity in control) × 100

(Eq. 2)

Colour measurement

The colour of all mulberry juice samples was measured using DC-P3 Automatic Colour Difference Meter (Beijing Xingguang Colour-Measurement Instrument Company Ltd., Beijing, China). The colour meter was calibrated using the white and black ceramic tiles provided. After calibration, a quartz cuvette was filled with the juice and duplicate readings taken. Hunter colour parameter of lightness coordinate (L*) gives a measure of the whiteness value, ranging from black (0) to white (100). The chromaticity coordinate (a*) ranges between red (+ve) and green (-ve). b* measures the colour parameter yellow (+ve) and blue (-ve). The hue angle (h°), chroma (C*), and total colour difference (ΔE) were respectively calculated using Equations 3 to 5 (Gimenez *et al.,* 2001):

$$h° = \arctan \frac{b*}{a*} \qquad \text{(Eq. 3)}$$

$$C* = \sqrt{a*^2 + b*^2} \qquad \text{(Eq. 4)}$$

$$\Delta E = \sqrt{(L* - L_o*)^2 + (a* - a_o*)^2 + (b* - b_o*)^2} \qquad \text{(Eq. 5)}$$

where, L_o*, a_o*, and $b_o* = $ control values obtained for untreated mulberry juice.

Microbiological count determination

Plate count agar (for total viable count) and oxytetracycline glucose yeast agar (for yeast and mould count) were used for initial and surviving microbial population enumeration (Benson, 1994). Serial dilution and the pour-plate method were employed. All the determinations were done in duplicates and microbial populations determined as CFU/mL of mulberry juice.

Statistical analysis

Analysis of variance (ANOVA) of mean values obtained for duplicate determinations of all variables determined were statistically analysed using SPSS 20.0 statistical package (SPSS Inc. Chicago, IL, USA) to determine whether significant differences ($p \leq 0.05$) existed among samples. Where significant differences existed, Duncan's least significance difference (LSD) test was applied to indicate where the differences existed.

Results and discussion

Effect of HHP on enzyme activity of mulberry juice

The mean RA of PPO and POD are shown in Figure 1. Sample A had the least PPO RA while sample I had the highest. There was a positive correlation between PPO RA and increases in pressure and time. A correlation analysis conducted gave a Pearson R value of 0.947 with *p*-value 0.000. This shows a strong positive relationship existing between PPO and increases in pressure and time. Since the *p*-value (0.000) $\leq$ 0.05, it can therefore be concluded that the relationship was statistically significant and that an increase in pressure and time led to an increase in PPO activity. Data obtained by Cao *et al.* (2011) also indicated higher resistance of PPO in strawberry to HHP treatment, although they recorded progressive inactivation at 400, 500, and 600 MPa in that order. A 95% reduction in PPO activity was reported by Palou *et al.* (1997) for banana puree with pH 3.4 and treated at 689 MPa. Dalmadi *et al.* (2006) also showed that PPO extracts of strawberry was stable until 500 - 700 MPa for 15 min, and at 800 MPa, PPO RA decreased to only 5%.

In the present work, PPO activity was inactivated to a higher degree at lower pressures than at higher pressure levels. The activity of PPO varies in different food materials based on intrinsic factors as well as the treatment method employed (Buckow, 2009; Misra *et al.,* 2016). Pressure resistant
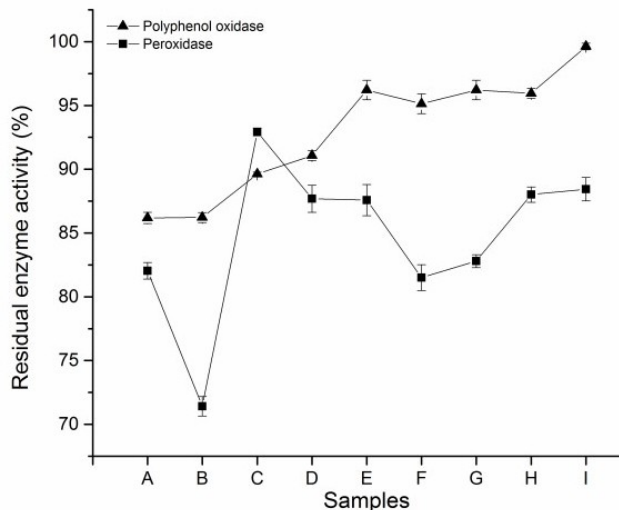
Figure 1. Residual enzyme activity of high hydrostatic pressure-treated mulberry juice. A = 200 MPa/10 min; B = 200 MPa/20 min; C = 200 MPa/30 min; D = 400 MPa/10 min; E = 400 MPa/20 min; F = 400 MPa/30 min; G = 600 MPa/10 min; H = 600 MPa/20 min; I = 600 MPa/30 min.

isoenzymes have been noted to be responsible for the final RA in food materials (Bayındırlı *et al.,* 2006). This may explain the differences in inactivation of mulberry juice PPO as compared to PPO from other sources.

There was no uniform trend in the inactivation pattern of POD RA as compared to PPO RA. At 200 MPa, POD RA was reduced after 10 min from 100% to 82.04% and further reduced to 72.42%. However, HHP treatment at 200 MPa for 30 min had a relatively lower effect, leading to 92.94% retention of POD activity. There was no significant difference ($p > 0.05$) in the POD RA at 400 MPa for 10 and 20 min. POD RA, however, dropped after 30 min. At 600 MPa, POD RA was significantly ($p \leq 0.05$) lower after 10 min than at 20 and 30 min. The increase in POD RA after 20 min could be due to pressure activation of latent forms due to conformational changes of the enzyme (Akyol *et al.,* 2006). However, there was no significant difference ($p > 0.05$) at 600 MPa for 20 and 30 min. Akyol *et al.* (2006) found POD RA of sliced carrot to decrease to 16% at 350 MPa and 20°C for 30 min, but an increase in the activity occurred with increasing pressure of over 350 MPa. Other studies observed a similar trend of POD RA in HHP treated strawberry juice and orange juice (Cano *et al.,* 1997; Cao *et al.,* 2011).

Effect of HHP on anthocyanin content of mulberry juice

ANOVA results obtained for per cent retained anthocyanins are displayed in Table 2.

Anthocyanin content of HHP-treated mulberry juice samples were affected to different levels. Sample A was least affected by HHP treatment and therefore retained the highest per cent anthocyanin, while sample D was most affected and retained the least per cent anthocyanin. Significant differences ($p \leq 0.05$) existed in per cent anthocyanin retained among most of the samples, with a few being not significantly different ($p > 0.05$) as shown in Table 2. The lowest pressure (200 MPa) employed caused less destruction on anthocyanins while application of higher pressures relatively led to higher losses. This observed phenomenon could be attributed to the higher activity of PPO and POD at higher pressures.

Table 2. Anthocyanin (%) retained in high hydrostatic pressure-treated mulberry juice.

Sample	Anthocyanin retained (%)
A	96.50 ± 0.74[g]
B	89.96 ± 0.37[d]
C	93.80 ± 0.62[f]
D	84.50 ± 0.68[a]
E	90.32 ± 0.37[d,e]
F	89.58 ± 0.32[c,d]
G	91.28 ± 0.25[e]
H	87.87 ± 0.37[b]
I	88.77 ± 0.32[b,c]

Mean values with different superscripts are significantly different. A = 200 MPa/10 min; B = 200 MPa/20 min; C = 200 MPa/30 min; D = 400 MPa/10 min; E = 400 MPa/20 min; F = 400 MPa/30 min; G = 600 MPa/10 min; H = 600 MPa/20 min; I = 600 MPa/30 min.

A correlation analysis to determine the extent to which PPO and POD activities influenced anthocyanin retention gave a Pearson R for PPO and POD as -0.425 and -0.075 with *p*-values of 0.079 and 0.767, respectively. This indicates that there was a moderately negative relationship that existed between the anthocyanin content of the mulberry juice and PPO activity, where lower PPO enzyme activities led to higher percent retention of anthocyanin, and *vice versa*. Also, there was a weak negative relationship between the anthocyanin content and POD activity. However, this relationship was not statistically significant ($p > 0.05$), meaning that an increase in the

Table 3. Colour characteristics of high hydrostatic pressure-treated mulberry juice.

Sample	L*	a*	b*	Hue	Chroma	*ΔE
A	5.92 ± 0.07^d	1.74 ± 0.18^e	4.72 ± 0.12^c	$69.84 \pm 1.39^{b,c}$	$5.03 \pm 0.06^{c,d}$	$1.30 \pm 0.22^{a,b}$
B	6.30 ± 0.07^e	3.92 ± 0.02^f	3.13 ± 0.04^a	38.64 ± 0.23^a	$5.01 \pm 0.04^{c,d}$	3.00 ± 0.04^e
C	$5.85 \pm 0.07^{c,d}$	1.64 ± 0.15^e	4.50 ± 0.50^c	$70.03 \pm 0.38^{b,c}$	$4.80 \pm 0.42^{b,c}$	1.10 ± 0.26^a
D	$5.76 \pm 0.05^{b,c}$	1.86 ± 0.03^e	4.60 ± 0.06^c	68.00 ± 0.03^b	$4.96 \pm 0.05^{c,d}$	$1.22 \pm 0.05^{a,b}$
E	5.69 ± 0.01^b	-0.07 ± 0.03^c	5.58 ± 0.09^d	90.74 ± 2.32^f	$5.58 \pm 0.09^{e,f}$	2.23 ± 0.04^c
F	6.72 ± 0.02^f	-0.62 ± 0.06^b	5.20 ± 0.21^d	96.80 ± 2.57^g	$5.24 \pm 0.18^{d,e}$	2.64 ± 0.06^d
G	6.65 ± 0.06^f	1.18 ± 0.06^d	5.55 ± 0.10^d	78.00 ± 0.35^e	5.67 ± 0.08^f	2.21 ± 0.11^c
H	6.76 ± 0.03^f	1.16 ± 0.02^d	4.40 ± 0.12^c	$75.18 \pm 0.13^{d,e}$	4.56 ± 0.11^b	1.52 ± 0.04^b
I	7.09 ± 0.06^g	-1.50 ± 0.19^a	7.40 ± 0.04^e	101.50 ± 1.47^h	7.54 ± 0.01^g	4.81 ± 0.06^f
Control	5.42 ± 0.06^a	1.10 ± 0.25^d	3.71 ± 0.10^b	$73.56 \pm 3.20^{c,d}$	3.88 ± 0.02^a	

Mean values with different superscripts within a column are significantly different. A = 200 MPa/10 min; B = 200 MPa/20 min; C = 200 MPa/30 min; D = 400 MPa/10 min; E = 400 MPa/20 min; F = 400 MPa/30 min; G = 600 MPa/10 min; H = 600 MPa/20 min; I = 600 MPa/30 min; *ΔE = Colour difference.

activity of POD does not necessarily lead to a corresponding reduction in anthocyanin retained. Activities of PPO and POD are known to catalyse the degradation of anthocyanins in food systems (Jackman *et al.,* 1987; Bodelón *et al.,* 2013). This could be the result of indirect oxidation by phenolic quinones generated by PPO and POD (Kader *et al.,* 1997; Skrede *et al.,* 2000).

For each pressure treatment level of 200, 400, and 600 MPa, treatment times of 10, 20, and 10 min gave the highest retention of anthocyanins in the samples, respectively. Degradation of mulberry juice anthocyanins observed in the present work contradicts the work by Garcia-Palazon *et al.* (2004) who reported that raspberry and strawberry anthocyanins were stable even up to 800 MPa/20°C/15 min. This may be due to the higher pressure adopted in their work which also inactivated PPO activity to a greater extent. Patras *et al.* (2009) recorded slight increases in anthocyanin content of purees of strawberry and blackberry after treatment at 400, 500, and 600 MPa, although they were not significantly different.

Effect of HHP on colour parameters of mulberry juice

For colour lightness (L*) of mulberry juice, significant increases ($p \leq 0.05$) were observed in the mean values obtained for treated samples when compared with the control (Table 3). There were significant differences ($p \leq 0.05$) among some of the samples in terms of lightness of their colour. Anthocyanins are the major pigments that give colour to most berries and other fruits (Steyn, 2009) and their destruction due to the RA of PPO and POD (Jiang *et al.,* 2004) may have accounted for the increased

colour lightness observed in treated samples. Sample I, which had the highest PPO RA also had the highest value for L*, indicating a positive correlation between residual activity of PPO and L*. A correlation analysis between PPO RA and L* gave a Pearson R value of 0.590 and *p*-value of 0.01, proving that a strong positive relationship existed between PPO RA and colour lightness, and that the relationship was statistically significant ($p \leq 0.05$). PPO and POD are known to degrade anthocyanins (Wrolstad *et al.,* 2005). Butz *et al.* (1994) made similar observation in HHP-treated mushrooms and onions.

There were significant differences ($p \leq 0.05$) in the a* values of treated samples when compared with the control, except samples G and H. Samples A to D had significantly higher a* values than the control sample, indicating improvement in the red colour of these samples. However, samples E, F, and I had negative a* values, signifying a shift from the red to the green colour region. It could be inferred from these results that lower pressures preserved the red colour of mulberry juice better than higher pressures. Bodelón *et al.* (2013) using pressures of 100 - 400 MPa, recorded insignificant difference in the a* value of pressurised and untreated strawberry puree.

For the chromaticity parameter of b*, all treated samples had significantly higher ($p \leq 0.05$) values when compared with that of the control sample, except sample B. Sample I had the highest b* value while sample B had the lowest. A similar observation was made by Bodelón *et al.* (2013).

The hue values of samples A to D were significantly lower ($p \leq 0.05$) than the control. However, samples E to I had significantly higher values

than the control. There were no significant differences among samples A, C, and the control. Sample I had the highest hue value, indicating the highest departure from the control. Hue angle is expressed on a 360° grid where 0° = bluish–red, 90° = yellow, 180° = green, and 270° = blue (Wrolstad *et al.,* 2005). Samples E, F, and I had hue values higher than 90°, therefore going beyond the red boundary.

For chroma, which indicates colour intensity, all treated samples were significantly different ($p \leq 0.05$) from the control. Samples C and H had mean values closer to the control than the others. Sample I had the highest mean chroma value, signifying that it had the lowest colour intensity among all the samples.

Total colour difference (ΔE) indicates the perceptible differences that exist between samples. Choi *et al.* (2002) have indicated that $\Delta E > 2$ corresponds to noticeable differences in the visual perception of many products. Samples A, C, D, and H had $\Delta E < 2$, meaning that there were no perceptible differences between these samples and that of the control. Sample I had the highest ΔE mean value, implying that, it had the highest colour deviation among the samples, relative to the control. Kong *et al.* (2003) reported that degradation of anthocyanin pigments correlates well with decreases in colour. Due to PPO and POD RA after HHP treatment, degradation of anthocyanins was likely to take place, explaining the colour differences among samples (Skrede *et al.*, 2000). This may explain why all the colour variables of sample I was highly affected as it had the highest PPO and POD RA.

Effect of HHP on microorganism of mulberry juice

The microbiological populations of HHP treated samples were inactivated to below detectable levels (< 10 CFU/mL) from the initial levels as shown in Table 4. Total viable count, mould, and yeast were reduced by $\log_{10}$ 4.86, 4.45, and 4.83, respectively. These results are similar to that obtained by Houška *et al.* (2006) in their work on apple-broccoli juice. Also, Ogawa *et al.* (1990) reported that treatment at 400 MPa at room temperature resulted in at least $\log_{10}$ 4 reduction in viability of conidia of *Aspergillus awamori* and sporangiospores of *Mucor plumbeus*. Moreover, Hocking et al. (2006) reported, after 60 s pressurisation of cultured cells in sucrose solution adjusted to pH 4.2 at 400 MPa, a $\log_{10}$ 3 to 4 reduction for both *Saccharomyces cerevisiae* and *Pichia anomala* was observed, as well as $\log_{10}$ 4 to 5 reduction after 120 s at 400 MPa. However, at 400 MPa and 120 s, no survivors were detected for *Penicillium expansum* and *Fusarium oxysporum*.

Microbial reductions after HHP treatment are therefore in line with other similar works.

Conclusion

In the present work, the levels of pressure and time used were able to retain anthocyanin content to between 84.50% and 96.50%. Comparatively, the inactivation of peroxidase was more pronounced than polyphenol oxidase activity for HHP-treated mulberry juice samples. The total viable count, mould, and yeast were reduced by $\log_{10}$ 4.86, 4.45, and 4.83 CFU/mL, respectively, making the juice safe for consumption. Comparatively, treatment at 200 MPa/10 min gave a better inactivation of PPO and POD, better retention of anthocyanin, and preserved the colour of the mulberry juice to almost the same extent as the control, making it the best treatment to be adopted.

Table 4. Results of microbial analysis of high hydrostatic pressure-treated mulberry juice samples.

Sample	Total viable count (CFU/mL)	Mould (CFU/mL)	Yeast (CFU/mL)
A	< 10	< 10	< 10
B	< 10	< 10	< 10
C	< 10	< 10	< 10
D	< 10	< 10	< 10
E	< 10	< 10	< 10
F	< 10	< 10	< 10
G	< 10	< 10	< 10
H	< 10	< 10	< 10
I	< 10	< 10	< 10
Control	5.3×10^4	6.8×10^4	9.8×10^5

A = 200 MPa/10 min; B = 200 MPa/20 min; C = 200 MPa/30 min; D = 400 MPa/10 min; E = 400 MPa/20 min; F = 400 MPa/30 min; G = 600 MPa/10 min; H = 600 MPa/20 min; I = 600 MPa/30 min.

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