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Effect of anthocyanins on lipid oxidation and microbial spoilage in value-added emulsions with bilberry seed oil, anthocyanins and cold set whey protein hydrogels

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ABSTRACT

The objective of this work was to explore the storage properties of a structured oil-in-water emulsion containing both water- and fat-soluble bioactive compounds from bilberries (*Vaccinium myrtillus* L.). Bilberry seed oil (BSO) was dispersed in a continuous aqueous phase of anthocyanins (AC) and whey protein isolate. The microstructure was evaluated using light microscopy and the effect of anthocyanins on lipid oxidation and microbial growth was investigated. The results showed that it was possible to generate a stable emulsion structure that resisted phase separation during 25 weeks of storage. Gas chromatography–mass spectrometry measurements of the fatty acids in the BSO during storage showed that AC had a protective effect against lipid oxidation. The AC did not have an antimicrobial effect against the investigated strains *Zygosaccharomyces bailii* (ATCC 42476) and *Aspergillus niger* (ATCC 6275 (M68)).

1. Introduction

Bilberries (*Vaccinium myrtillus* L.) are a natural source of water-soluble phenolic compounds such as anthocyanins and functional oils with a high content of polyunsaturated fatty acids (PUFA), high levels of tocopherols and a low n-6/n-3 ratio (Yang, Ahotupa, Määttä, & Kallio, 2011). Consumption of bilberries is associated with reduced risk of cardiovascular diseases (Basu, Rhone, & Lyons, 2010; Habanova et al., 2016), cancer (Marjorie et al., 2007) and diabetes (Asgary, Rafieian Kopaei, Saahebkar, Shamsi, & Goli- Malekabadi, 2016). As a consequence, the market for processed bilberry products and dietary supplements containing bilberry extracts has expanded significantly (Primetta, Jaakola, Ayaz, Inceer, & Riihinen, 2013). The industries that produce these products also generate by-products in the form of a press cake that still contains significant amounts of anthocyanins and berry seed oils (Paes, Dotta, Barbero, & Martínes, 2014). Previous research has demonstrated that extraction technologies can collect both the polar anthocyanins (Babova, Occhipinti, Capuzzo, & Maffei, 2016) and non-polar seed oils (Gustinelli, Eliasson, Svelander, Alminger, & Ahrné, 2017; Laroze, Diaz-Reinoso, Moure, Zuniga, & Dominguez, 2010) from dried bilberry press cakes. In order to achieve improved use of raw materials and increase the value of all material from the berry industry, it is desirable to develop food products containing multiple bioactive compounds from bilberry press cakes. Such products might include emulsions systems with both water-soluble anthocyanins and non-polar berry seed oils.

Anthocyanins have been reported to contribute to the extension of shelf-life in emulsions by acting as an antioxidant and thus preventing lipid oxidation in liquid oil-in-water emulsions containing fish oil (Zhang, Shen, Prinyawiwatkul, King, & Xu, 2013) or soy bean oil (Li, Kim, Li, Lee, & Rhee, 2014). Moreover, anthocyanins have been reported to inhibit the growth of Gram-positive, Gram-negative bacteria and mould in disc diffusion tests (Demirbas, Yilmaz, Ildiz, Baldemir & Ocsoy, 2017; Trikas, Melidou, Papi, Zachariadis, & Kyriakidis, 2016) as well as on whole fruits (Sivankalyani, Feygenberg, Diskina, Wright, & Alkan, 2016). However, to the best of our knowledge there are no investigations of the antimicrobial effect as well as protective effect against lipid oxidation of anthocyanins in more complex and processed food matrixes.

Furthermore, designing functional food emulsion systems with both anthocyanins and bilberry seed oil is challenging as they are prone to chemical degradation, with subsequent loss of biological activity. Anthocyanins are sensitive to UV light, temperature and oxygen (Clifford, 2000), and are more stable in acidic environments (pH ≤ 3)

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(Cabrita, Fossen, & Andersen, 2000). Attempts to stabilize pure extracted anthocyanins have used various matrices based on polysaccharides (Chung, Rojanasasithara, Mutilangi, & McClements, 2015; Ferreira, Faria, Grosso, & Mercadante, 2009; Xiong, Melton, Easteal, & Siew, 2006) or whey proteins (Betz & Kulozik, 2011; Betz et al., 2012; Viljanen, Kylli, Ubbermann, Schwarz, & Heinonen, 2005). Its high PUFA content makes berry seed oil highly sensitive to lipid oxidation. This may be avoided by gently trapping the oil in a liquid oil-in-water emulsion system together with an antioxidant in the continuous phase (Niki, Yoshida, Saito, & Noguchi, 2005).

The objective of this work was to investigate the microstructure together with the effect of anthocyanins on lipid oxidation and microbial growth in a structured oil-in-water emulsion with bilberry seed oil dispersed in a continuous phase of anthocyanins and cold set whey protein gel.

2. Materials and methods

2.1. Materials

Anthocyanins (AC) were extracted from bilberry press cake (Svantes Vilt and Bär, Harads, Sweden) using the technique described by Eliasson, Labrosse, and Ahrne (2017), freeze dried and re-dissolved in a citric acid buffer at pH 3 in order to create a stock solution. The concentration of AC in the stock solution was measured spectrophotometrically with a pH differential method (AOAC, 2005) and was found to be 1351 μg/ml. The bilberry seed oil (BSO) used was BLUE TOCOL Bilberry Seed Oil (Bionord Biokemi AB, Stenugnsund, Sweden), the whey protein isolate (WPI) used was BiPRO™, (Davisco Foods International, Inc., Le Sueur, MI, USA), and D-(+)-Gluconic acid d-lactone (GDL) (Sigma-Aldrich, St Louis, MO, USA) was used to reduce pH in the emulsions. One emulsifier was used: Tween 80 (Merck-Schuchardt OHG, Hohenbrunn, Germany). The methoxysolution used for transesterification was prepared by mixing 1.1 sodium with 100 ml methanol using a magnetic stirrer.

2.2. Emulsion preparation

The emulsions were prepared according to the following protocol: A stock solution of 12% w/w BiPRO™ WPI was dissolved in distilled water and kept at 68.5 °C while stirring for 2 h in order to create reactive WPI aggregates (de Jong & van de Velde, 2007). The WPI stock solution was then allowed to cool to 20 °C before diluting with 20% (w/w) of AC solution together with 1% (w/w) of Tween 80 using a magnetic stirrer. After diluting, the resulting WPI concentration was 9.6% (w/w) and the resulting concentration of AC was 256 μg/ml. Pure citric acid buffer solution at pH 3 (20% w/w) was used as reference. Gelation was induced by reducing the pH by adding 1.1% or 7% (w/w) of GDL in order to reach a final pH of 4.5 or 3 respectively. The reduction in pH versus time was recorded using a pH meter pH1100L (VWR International, Radnor, USA). Directly after adding the GDL to the AC-WPI solution, 37.5% w/w of BSO was added and mixed using an Ultra-Turrax (Skafte MedLab AB, Onsala, Sweden) at 24,000 rpm for 30 s. Table 1 shows the WPI:AC ratio, reference samples, amount of added GDL, resulting pH and analyses performed during storage.

2.3. Microstructure characterization during storage

The microstructure of the emulsions (samples 1–8 in Table 1) after 25 weeks of storage at 20 °C and 26 °C was evaluated with a light microscope (LM) Nikon Microphot-FXA (Japan) to which an Altra 20 camera and a computer were connected. The samples were analyzed using 10×, 20×, 40× and 100× objectives and micrographs collected with 40× was used for the results presented in this work as it enabled the droplets and colour of the continuous phase to be identified clearly. The emulsions were smeared out on object glasses and studied directly under the microscope without any staining. White balance of the background was adjusted before taking the images in order to be able to make comparisons of the AC colour in the different samples.

2.4. Oxidation of BSO during storage

Emulsions were prepared according to Section 2.2 with the addition of 0.02% (w/w) sodium azide to the continuous phase as microbial preservative. Subsequently, two replicates (1.5 ml) of all samples listed in Table 1 were transferred to Eppendorf tubes and stored in sealed tubes for 25 weeks at 20 °C and 26 °C. Two Eppendorf tubes containing 1.5 ml of pure BSO were kept in both conditions as reference. After storage, approximately 0.015 g of each sample was collected twice, and the lipid phase was extracted by mixing with 1 ml hexane + C19-TAG (SIGMA) followed by ultrasonication and shaking. The hexane solution containing the BSO was then further washed with 1 ml of water. Thereafter, 200 μl of the solution was collected and transesterified by mixing with 200 μl methoxysolution for 30 min followed by centrifugation. The hexane phase was collected and analysed by gas chromatography–mass spectrometry (GC/MS). The fatty acid methyl esters (FAME) were analysed using a general FAME-gradient by separation on a 5% phenylmethylsiloxane column and a temperature gradient from 35 to 320 °C with a rate of 8 °C/min in the temperature range of interest.

To quantify the lipid oxidation, the chromatograms for each sample replicate were integrated and normalized to the C19-FAME resulting from the transesterification of the C19-TAG used as reaction standard. Thereafter the lipid oxidation was quantified by the following formula: C16:0/(C18:1 + C18:2), which corresponds to the ratio between the area of saturated palmitic acid, C16:0 and the sum of the area of the unsaturated linoleic (C18:2) and linolenic acids (C18:3) as pre-trials displayed that these constituted the main fatty acids in the BSO and were affected by lipid oxidation during storage.

2.4.1. Statistical analysis

The ratio between C16:0 and sum of C18:1 and C18:2 was calculated for both replicates of the samples listed in Table 1 and standards deviation for the two replicates were calculated. In addition, in order to further evaluate if there was any statistical difference between emulsions with or without AC on lipid oxidation, the following procedure was performed:

i. The C16:0/(C18:1 + C18:2) ratio for all replicates containing AC where summarized (sample 1–4 in Table 1)
ii. The C16:0/(C18:1 + C18:2) ratio for all replicates without AC were summarized (sample 5–8 in Table 1)., a
iii. A *t*-test was performed on the resulting averaged ratios from (i) and (ii).

2.5. Microbial test

2.5.1. Microorganisms

Two different microorganisms were used as test organisms in the present study, *Z. bailii* (ATCC 42476) and *A. niger* (ATCC 6275 (M68)). The test organisms were inoculated in separate samples and not as a mixed culture. Cultures were maintained using a microbial cryopreservation system (Mast Cryobank™) at −20 °C.

2.5.2. Inocula

A 10 ml culture of *Z. bailii* was prepared in yeast extract peptone dextrose (YPD) broth and incubated aerobically at 30 °C for 18 h. The initial concentration of *Z. bailii* was 10^6 colony-forming units (CFUs) ml^{-1}. The culture of *A. niger* was grown on Malt Extract Agar (MEA) for at 21 ± 1 °C for 11 days. Spores were harvested using a solution of NaCl 0.9% (w/v) with 0.05% Tween 80 (v/v) (Acros Organics, CAS 9005-65-6) and counted in a Bürkers chamber. The final

Table 1
Composition of the different emulsions i.e. the WPI:AC or WPI: pure citric acid buffer ratio, amount of added GDL, resulting pH, storage temperature and analyses performed during storage.

Sample no	Sample composition WPI:AC in buffer ratio	References WPI:pure buffer ratio	Amount of GDL (w/w)	Final PH	Storage temp (°C)	Evaluation during storage		
						Microbial stability	Lipid oxidation	Micro-structure
1	80:20		7.0	3.0	21	No	YES	YES
2	80:20		1.1	4.5	21	YES	YES	YES
3	80:20		7.0	3.0	26	No	YES	YES
4	80:20		1.1	4.5	26	No	YES	YES
5		80:20	7.0	3.0	21	No	YES	YES
6		80:20	1.1	4.5	21	YES	YES	YES
7		80:20	7.0	3.0	26	No	YES	YES
8		80:20	1.1	4.5	26	No	YES	YES

concentration for the spore suspension was approximately 10^5 spores ml^{-1}. The spore suspension was kept at 8 °C overnight.

2.5.3. Sample preparation

Emulsions at pH 4.5 (samples 2, 4, 6 and 8 in Table 1) were prepared, according to Section 2.2, under aseptic conditions and transferred to polypropylene tubes (100 × 16 mm, Sarstedt), 3 ml emulsion per tube. A total of 120 polypropylene tubes were prepared. 40 tubes of emulsion with AC (sample 2 in Table 1) and 40 tubes of emulsion with pure citric buffer (sample 6 in Table 1) were inoculated with the test organisms, where sample 4 (Table 1) served as reference. The test organisms were inoculated in separate samples and not as a mixed culture. Inoculation concentration of *A. niger* spores was 10^3 spores ml^{-1} emulsion and *Z. bailii* was 10^4 CFU ml^{-1} emulsion.

An additional 40 polypropylene tubes were prepared with emulsion without anthocyanins (Sample 6 in Table 1). These samples were not inoculated with the test organisms and served as negative controls. For each sampling point, three replicate tubes with emulsion were prepared.

2.5.4. Microbiological analysis

Emulsions were homogenized within the polypropylene tubes by adding 3 ml 0.1% peptone saline. Subsequently, emulsions were finely divided with a sterile spatula followed by addition of sterile glass beads to the tubes before final homogenization by vortex for 2 min. From the homogenate, serial dilutions were prepared in 0.1% peptone saline, varying from 10^{-1} to 10^{-6}. The dilutions were used to determine counts of *Z. bailii* and *A. niger*. Organisms were recovered using the following selective media: *Z. bailii*, aerobic, Oxytetracycline Glucose Yeast Extract (OGYE) agar, enumerated after 48 h incubation at 25 °C; *A. niger*, aerobic, MEA, enumerated after 3–6 days incubation at 22 °C.

Each sample (inoculated sample 2, inoculated sample 4 and non-inoculated reference) were used as zero reference samples and were held at 21 ± 1 °C and sampled within the next 4 h. Initial pH (HI 2210 pH Meter HANNA instruments) and water activity (a_w) (CX-2 Water Activity Meter, Aqua Lab) were measured in three sample replicates from each emulsion preparation (inoculated -, reference – and negative control emulsion).

All samples were stored at 22 °C. Sampling was conducted every third day for *Z. bailii* and weekly for *A. niger*, with a total of four sampling points after inoculation. For each sampling point, three sample replicates from each emulsion preparation were collected and analysed.

Cell counts were converted into decimal logarithm values to illustrate the growth curves of *Z. bailii* (12 days of storage) and *A. niger* (4 weeks of storage), Fig. 3. Plotted data in Fig. 3 are the mean of three replicates. Standard deviation of each sampling point was determined based on three replicates and their respectively logarithmic values.

3. Results and discussion

3.1. Structure development during storage

To establish that the developed emulsion systems were stable during the entire investigated storage period of 25 weeks, the microstructure was evaluated by LM. Fig. 1 presents micrographs of emulsions at pH 3 and pH 4.5 with and without AC (samples 1–2 and 5–6 in Table 1) stored at 21 °C.

The results in Fig. 1 depict the continuous phase of AC and WPI or pure citric acid buffer and WPI surrounding the BSO droplets. The results clearly demonstrate that although the BSO droplets had agglomerated, no phase separation had occurred, and the emulsions were still intact after 25 weeks of storage. Similar results were achieved for samples stored at 26 °C (not shown). The samples on the left in Fig. 1 (a and c) both contain AC, which appears as purple fields in the LM micrographs. The purple colour was more pronounced in the continuous phase of samples at pH 3 (Fig. 1(a)). This can be explained by the improved stability of anthocyanins at pH 3 (Cabrita et al., 2000). Furthermore, AC consists of multiple species that are highly dependent on pH. At strong acidic conditions (pH ≤ 1) the predominant AC is the red-coloured flavylium cation while at higher pH ≥ 4 the uncharged and relatively unstable blue quinonoidal species is in the majority (Cabrita et al., 2000). This was also visible in the developed emulsions where samples at pH 3 were almost reddish in colour while samples at pH 4.5 were significantly bluer/purple.

3.2. Oxidative stability of BSO

The oxidation of BSO during storage was estimated by extracting the oil from the emulsions and measuring its fatty acid profile using GC/MS. A ratio was calculated by dividing the peak areas for palmitic acid (C16:0) by the sum of the mono- and polyunsaturated oleic (18:1) and linoleic (C18:2) acids as pre-trails displayed that as lipid oxidation proceeded during storage the quantities of the unsaturated fats C18:1 and C18:2 was reduced while amounts of saturated C16:0 increased. Thus, a large ratio equals to higher levels of lipid oxidation. The ratios for all samples (samples 1–8 in Table 1) are illustrated in Fig. 2.

The results in Fig. 2 all display the same trend: samples containing AC show a lower ratio of saturated to unsaturated oils, which correlates with reduced lipid oxidation in these samples. However, as can be seen by the size of the standard deviations on the bars in Fig. 2, no significant statistical difference could be determined within the four different sample groups (pH 3 and 4.5 at 21 or 26 °C respectively) . However, as the trend with reduced lipid oxidation in emulsions with AC was the same in all sample groups, an additional statistical evaluation was conducted where the ratio for all samples containing AC (sample 1–4 in Table 1) was summarized and compared by a *t*-test to the summarized ratios of all samples without AC (sample 5–8 in Table 1). The *t*-test showed that the ratios for all samples with AC were

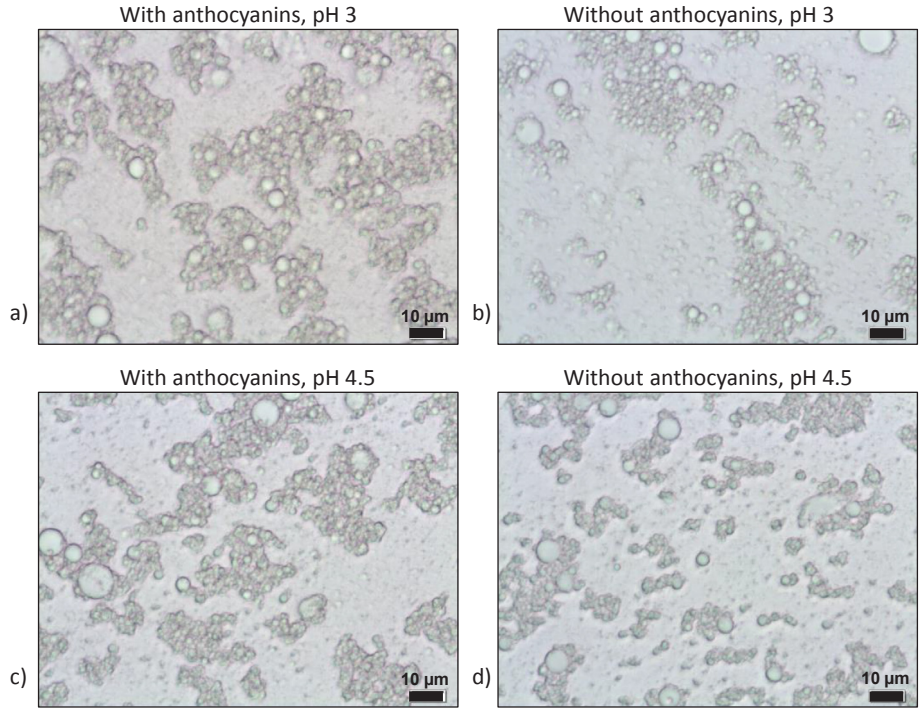

Fig. 1. LM micrographs of emulsions after 25 weeks of storage at 20 °C. Emulsion systems at pH 3 in continuous AC + WPI phase (a) and pure citric acid buffer (b) as well as emulsion systems at pH 4.5 in continuous AC + WPI phase (c) and pure citric acid buffer (d). Scale bars equal 10 µm.

significantly different from all samples without AC at a 95% confidence interval.

One could therefore postulate that the AC in the developed food emulsion systems has an inhibitory effect on the oxidation of the dispersed BSO. This result agrees with previous studies on the inhibitory effect of AC in food systems such as mayonnaise with soybean oil (Li et al., 2014) or liquid model o/w emulsions containing fish (Zhang, Butelli, et al., 2013). In general, lipids are oxidized by three distinct mechanisms; (i) enzymatic oxidation, (ii) non-enzymatic, free radical-mediated oxidation, and (iii) non-enzymatic, non-radical oxidation (Niki et al., 2005). The protective effect of AC against lipid oxidation has mainly been attributed to binding of free radicals i.e. prevention of mechanism (ii) mentioned above (Viljanen, Kylli, Kivikari, & Heinonen, 2004). Furthermore, lipid oxidation is accelerated by exposure to air, light or heat during processing (Alamed, McClements, & Decker, 2006). By applying a non-thermal emulsification method combined with structural stabilization of the continuous phase using WPI, which protects the oil from air and light exposure, and addition of AC, this work strived towards creating a optimal environment to preserve the original composition of the BSO.

AC are more stable at pH ≤ 3 (Cabrita et al., 2000), as was confirmed by the microstructure analysis in this study, where the samples

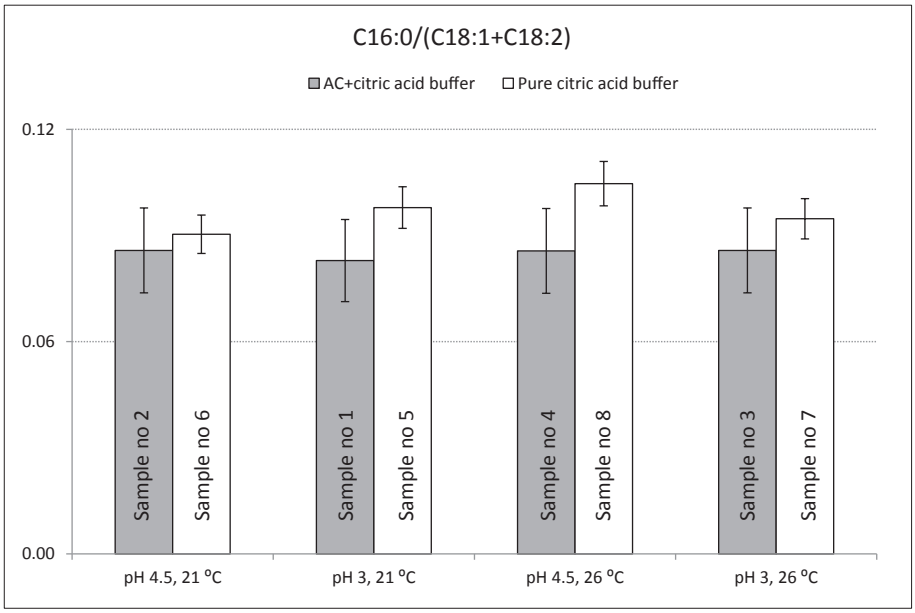

Fig. 2. Estimation of oxidation of BSO, using the ratio between the saturated oil (C16:0) and the sum of unsaturated oils (C18:1 and C18:2). A larger ratio corresponds to more oxidation. The error bars correspond to standard deviation of two sample replicates.

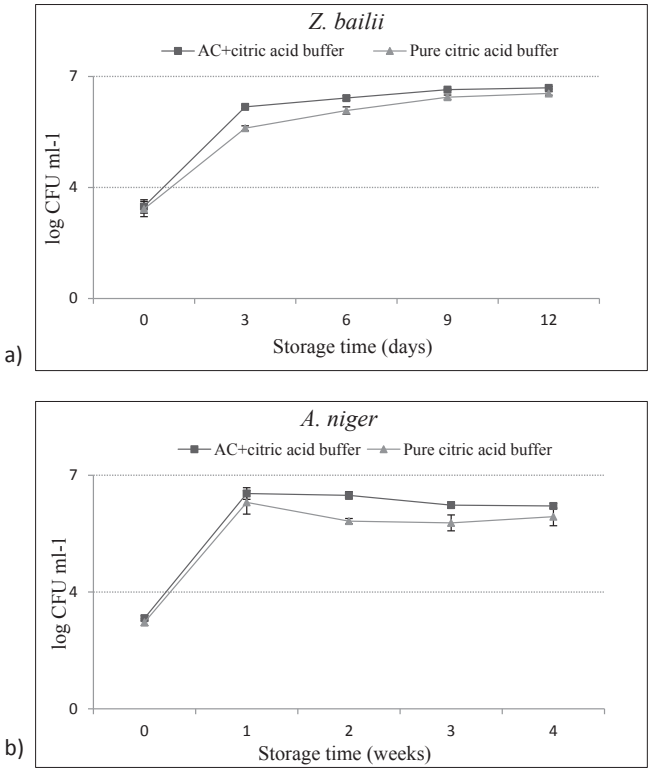

Fig. 3. Microbial growth in inoculated emulsions during storage at 22 °C expressed as log CFU per ml sample. The growth of a) *Z. bailii* or b) *A. niger* with AC and citric acid buffer or in pure citric acid buffer is shown. Data are the mean of three replicates and error bars represent the standard deviation of three replicates.

at pH 3 were more purple in the micrographs (Fig. 1(a))., It would therefore have been expected that the samples having pH 3 and containing AC would exhibit less lipid oxidation than corresponding samples at pH 4.5 , i.e. comparing sample no 1 with 2 and sample no 3 with 4 in Fig. 2. However, no such difference can be observed in Fig. 2. One possible explanation for this could be that the WPI gel strength is significantly weaker at pH 3 than at pH 4.5 (Alting, de Jong, Visschers, & Simons, 2002) and a rigid WPI gel has itself been reported to structurally inhibit lipid oxidation (Owens, Griffin, Khouryieh, & Williams, 2018) as well as to stabilize AC (Betz & Kulozik, 2011; Betz et al., 2012). Therefore, the structural stabilization exhibited by the WPI could compensate for the less favourable environment at pH 4.5 for the AC during storage.

3.3. Microbial growth

The ability of the developed emulsions to resist microbial growth was evaluated by inoculating them with *Z. bailii* and *A. niger*. The two test organisms were chosen as the antimicrobial effect of AC on Gram-positive and Gram-negative bacteria has recently been displayed (Demirbas et al., 2017; Tian et al., 2017; Trikas et al., 2016), whereas, to the best of our knowledge, the effect of anthocyanins on yeast and mould has not been investigated in processed food emulsion matrixes. The microbial growth during storage in terms of log CFU ml^{-1} for *Z. bailii and A. niger* respectively is presented in Fig. 3.

The growth curve for *Z. bailii* in Fig. 3(a) shows that after three days of storage at 22 °C, the growth for the control samples (samples with pure citric acid buffer) increased by 2 log CFU per ml, compared to an increase of 3 log CFU per ml for the samples with AC. Thereafter, the difference in microbial growth decreased between the two emulsion systems. After nine days of storage, the values were constant at 6 log CFU per ml in both systems. The microbial growth in emulsions inoculated with *A. niger* also displayed differences between samples with and without AC (Fig. 3(b)). Initially all samples exhibited similar microbial growth and rose to 6 log CFU per ml within the first week of storage. However, the control samples then dropped approximately 1 log CFU per ml at week 2, while the population remained rather constant (approximately 6 log CFU per ml) in samples with AC (Fig. 3(b)).

Consequently, emulsions inoculated with AC had more pronounced growth during storage of both *Z. bailii* and *A. niger* compared to control samples with pure citric acid buffer. For emulsions inoculated with *A. niger*, the difference was even visually apparent (Fig. 4).

In contrast to previous reports on bacteria (Demirbas et al., 2017; Tian et al., 2017; Trikas, et al., 2016) as well as fungi on whole fruits or vegetables (Sivankalyani, et al., 2016; Zhang, Butelli, et al., 2013; Zhang, Shen, et al., 2013), no antifungal effect was displayed by the addition of AC. It rather seemed that the yield of both *A. niger* and *Z. bailii* increased within emulsions with AC. However, the increased yield of *A. niger* in the AC broth may be solely a reflection of increased sporulation, notable in the top black fraction on the emulsion. Hence, it is possible that the level of growing spores is higher in submerged parts of the emulsion. Noteworthy is that *A. niger* and other spoilage moulds are generally spread by synthesis and dispersal of airborne spores indicating that increased spore synthesis is indeed problematic from a food safety perspective. Moreover, the increased fungal yield was also observed growth for the spoilage yeast *Z. bailii* indicating other growth promoting properties. During the experimental setup, all emulsions were prepared from the same WPI stock solution and no differences in pH or water activity was observed. Furthermore, the anthocyanin stock solution (Section 2.1) was spread on selective media used for the test organisms. No microbial growth was detected within the anthocyanin

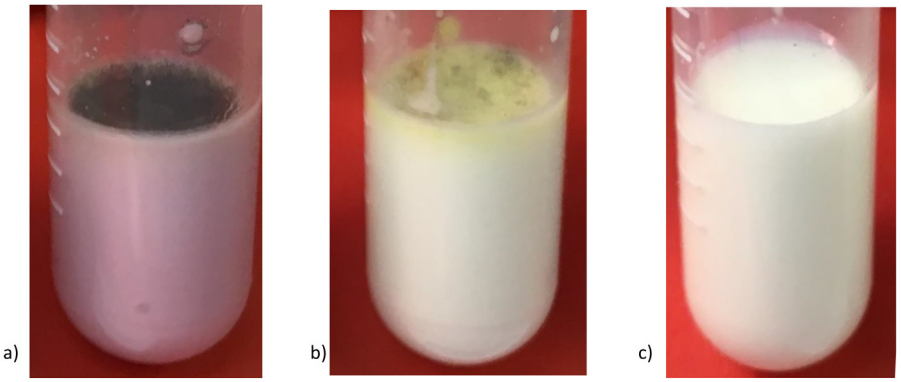

Fig. 4. Emulsions with (a) AC and (b) with pure citric acid, both inoculated with *A. niger* in polypropylene tubes, stored at 22 °C for 3 weeks. (c) Non-inoculated reference samples with citric acid buffer. The top of the emulsions demonstrate the growth of *A. niger*.

stock solution. Currently we have no explanation as to why the addition of AC, in our experiments, promotes fungal growth. The observed antimicrobial activities of AC in other studies may be explained both by the fact that different organisms were tested and, likely, in combination with different experimental setups. Nevertheless, our results indicate that there are situations where AC does not have any decisive antifungal activity. To elucidate any growth or sporulation inducing properties of AC further studies are required.

4. Conclusions

This study has shown that it is possible to create stable food emulsions containing both water and fat-soluble bioactive compounds from bilberry by-products by dispersing BSO in a continuous phase of AC stabilized by WPI. Although the BSO started to agglomerate during storage, the structure was stable enough to withstand phase separation. The AC was better preserved in the emulsions at pH 3 in the continuous phase. The AC displayed a protective effect against lipid oxidation of the BSO, both at pH 3 and pH 4.5. With the used experimental protocol, AC did not have any antifungal effect on *Z. bailii* and *A. niger* indicating that addition of AC may not be a sufficient preservation strategy of its own.

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Declaration of interest

Declarations of interest: None.

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