Inhibition of *Shewanella* spp. growth by *Syzygium australe* and *Syzygium luehmannii* extracts: natural methods for the prevention of fish spoilage

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Abstract *Syzygium australe* and *Syzygium luehmannii* fruit and leaf were investigated for their ability to inhibit *Shewanella* spp. growth. Extracts of both *Syzygium* spp. displayed potent growth inhibitory properties against all *Shewanella* spp. tested in disc diffusion and liquid diffusion assays. In general, *S. australe* extracts were more potent inhibitors of *Shewanella* spp. growth, and the fruit extracts were generally better than the corresponding leaf extracts. The methanolic *S. australe* fruit extract was a particularly potent inhibitor of all *Shewanella* spp. growth, with MIC values as low as 87 µg/mL. The aqueous and ethyl acetate *S. australe* fruit extracts were similarly potent inhibitors of *Shewanella* spp. growth, albeit with slightly higher MIC values. Several other *Syzygium* spp. extracts also were potent bacterial growth inhibitors, albeit with MIC values generally >1000 µg/mL. The most potent *S. australe* fruit extracts were nontoxic in the *Artemia franciscana* bioassay, with LC₅₀ values substantially >1000 µg/mL. The potent bacterial growth inhibitory activity and lack of toxicity of the *S. australe* fruit extracts indicate their potential as natural fish and seafood preservatives.

Keywords Food spoilage · Microbial rancidity · *Shewanella* · Brush cherry · Riberry · Natural preservative · Natural products

Introduction

Food loss through spoilage is a major global problem, resulting in large amounts of wasted food, as well as a possibility of foodborne illnesses. Spoilage may result in a deterioration of flavour, visual appearance, texture or nutritional value of food, rendering it unpalatable and/or increasing the risks of diseases and food poisoning. From initial harvesting through to handling and processing, food products are exposed to a variety of abiotic (temperature, heat, oxygen) and biotic elements (yeasts, fungi, insects, bacteria) (Ray and Bhunia 2008). Though spoiled food may be hazardous to human health if consumed, often putridity is simply aesthetic and the food is completely safe for consumption. However, the resultant undesirable odours, tastes or changes in texture results in an unfavourable product which is subsequently discarded.

Microbial spoilage is a major contributing factor to food deterioration, particularly in perishable foods, accounting for an estimated 25% of all food wastage (Ghaly et al. 2010). Spoilage of this type can be induced through either the introduction of foreign microbes as a result of improper handling/storage techniques, or through the proliferation of pre-existing microbes when conditions are favourable for growth (Ray and Bhunia 2008). Whilst many bacterial species may contribute to the spoilage of fish and other seafood, *Shewanella* spp. are generally acknowledged as a major cause of spoilage as they are psychrotolerant, grow both aerobically and anaerobically, and tolerate a wide pH range (Ng et al. 2015). Thus, they are often relatively unaffected by physical preservative methods which may limit the growth of other bacteria. The genus *Shewanella* are found in both freshwater and marine environments, often as part of fish microbial flora, the genus has been widely investigated due to their ability

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to utilise a range of electron acceptors, including trimethylamine-N-oxide (TMAO), a common osmolyte in marine fish (Boskou and Debevere 1998). Under anoxic conditions, *Shewanella* spp. are capable of utilising TMAO as an alternative to oxygen. Coupled with an appropriate electron donor, TMAO is reduced into trimethylamine (TMA), producing the “fishy aroma” commonly associated with spoilage (Boskou and Debevere 1998). Although *Shewanella putrefaciens* has long been considered the primary spoilage bacterium of stored marine fish, studies have revealed that *Shewanella baltica* and a number of other *Shewanella* species may also contribute to fish spoilage (Vogel et al. 2005).

The psychrophilic and psychrotrophic nature of many *Shewanella* spp., as well as their ability to grow under anoxic conditions, limits the effectiveness of storing fish products on ice or under anaerobic conditions. Some chemical treatments are successful in food spoilage management. However, customers are often concerned by the potential health risks associated with synthetic chemical compounds (Ray and Bhunia 2008). Investigating natural plant based preparations with known antimicrobial properties offers a safe and effective means of preventing bacterially-driven fish spoilage. Antimicrobial plant extracts with high antioxidant contents are particularly attractive as they may block oxidation of fish macromolecules, as well as inhibiting microbial growth and thus have pleuripotent preservative effects. Recent studies have demonstrated the potent bacterial growth inhibitory activity of several high antioxidant fruits and herbs against a wide panel of food spoilage and pathogenic bacteria (Sirdaarta et al. 2015; Winnett et al. 2014).

Syzygium is a large genus of evergreen flowering plants (family Myrtaceae) consisting of approximately 500 species (Page and Olds 2004). *Syzygium* spp. are widespread, occurring in tropical and subtropical regions of South-East Asia, Australia and Africa. Many species produce edible fruits and berries with high antioxidant contents. In the commercially most important species *Syzygium aromaticum* (clove), the unopened flower bud is used as a spice. This plant also has uses in traditional medicine as an anaesthetic and for its antibacterial activity (Cai and Wu 1996). Numerous studies have reported on the ability of *S. aromaticum* oils to inhibit the growth of panels of bacteria (Arora and Kaur 2007) and fungi (Park et al. 2007). South East Asian (*Syzygium jambos*) (Mohanty and Cock 2010), Indian (*Syzygium lineare* and *Syzygium cumini*) and African species (*Syzygium cordatum*) (Cock and van Vuuren 2015) also have potent antimicrobial activity. Much of our knowledge about the antiseptic properties of Australian *Syzygium* spp. is anecdotal, although Australian Aborigines are known to have used some species as medicinal agents (Cock 2011).

Extremely high antioxidant capacities have been reported for several Australian *Syzygium* spp. (Jamieson et al. 2014). Subsequent studies have examined the bacterial growth inhibitory properties against several Australia *Syzygium* spp. (Cock 2012; Chikowe et al. 2013). In particular, high antioxidant *Syzygium australe* (J. C. Wendl. Ex Link) B. Hyland (bush cherry) and *Syzygium leuhmannii* (F. Muell.) L. A. S. Johnson (riberry) extracts have been highlighted as potent inhibitors of a broad spectrum of bacteria (Cock 2012). Thus, treatment of fish with *S. australe* and/or *S. leuhmannii* extracts has the potential to inhibit both oxidative rancidity and microbial spoilage. Despite this, the *Shewanella* spp. growth inhibitory properties of *S. australe* and *S. leuhmannii* are yet to be evaluated. The current study was undertaken to examine the antiseptic potential of *S. australe* and *S. leuhmannii* fruit extracts against several species of *Shewanella*, and thus to evaluate their potential to prolong the shelf life and palatability of fish.

Materials and methods

Plant source and extraction

Syzygium australe (J. C. Wendl. Ex Link) B. Hyland and *Syzygium leuhmannii* (F. Muell.) L. A. S. Johnson leaves and fruit were collected from verified trees in suburban regions of Brisbane, Australia. Voucher specimens of all specimens were deposited in the School of Natural Sciences, Griffith University, Australia. The plant materials were thoroughly dried using a Sunbeam food dehydrator and the materials stored at -30°C until required. Prior to use, the plant materials were thawed and ground into a coarse powder. Individual quantities (1 g) of the ground plant material were weighed into separate tubes and 50 mL of deionised water, ethyl acetate or methanol were added. All solvents were obtained from Ajax, Australia and were AR grade. The ground plant materials were extracted in each solvent for 24 h at 4°C by gentle shaking. The extracts were subsequently filtered through filter paper (Whatman No. 54) under vacuum and dried using rotary evaporation in an Eppendorf concentrator 5301. The resultant dried extracts were weighed and dissolved in 10 mL deionised water (containing 1% DMSO).

Qualitative phytochemical studies

Phytochemical analysis of the *Syzygium* spp. extracts for the presence of cardiac glycosides, alkaloids, saponins, tannins, flavonoids, phenolic compounds, phytosterols, flavonoids and triterpenoids was achieved as previously described (Courtney et al. 2015).

Antioxidant capacity

The antioxidant capacity of the samples was determined using a modified 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) free radical scavenging method (Jamieson et al. 2014). Ascorbic acid (0–25 µg per well) was used as a reference and the absorbances were measured at 515 nm. All tests were completed alongside controls and ascorbic acid standards on each plate and performed in triplicate. The antioxidant capacity based on DPPH free radical scavenging ability was assessed for each extract and expressed as mg ascorbic acid equivalents per gram of original plant material extracted.

Antibacterial screening

Environmental Shewanella strains

Shewanella putrefaciens strain 200, *Shewanella baltica* strain OS155, *Shewanella frigidimarina* strain NCIMB 400 and *Shewanella loihica* strain PV-4 used in this study were kindly donated by Professor Kenneth Nealson of the University of Southern California, United States of America. Antibacterial screening was achieved using a modified peptone/yeast extract (PYE) agar containing: 1 g/L peptone, 1.5 g/L yeast extract, 7.5 g/L NaCl, 1 g/L ammonium persulfate, 2.4 g/L HEPES buffer (pH 7.5) and 16 g/L bacteriological agar as previously described (Wright et al. 2016). The *S. putrefaciens* and *S. loihica* cultures were incubated at 30 °C for 24 h. The *S. baltica* and *S. frigidimarina* cultures were incubated at 15 °C for 72 h. All stock cultures were subcultured and maintained in PYE media at 4 °C. The media nutrient components were supplied by Oxoid Ltd., Australia.

Evaluation of antibacterial activity

Antibacterial activity screening of the *Syzygium* spp. extracts was assessed using a modified disc diffusion assay (Kalt and Cock 2014). Briefly, 100 µL of each individual *Shewanella* spp. was grown separately in 20 mL of fresh nutrient broth until an approximate count of 10^8 cells/mL was reached. A volume of 100 µL of each bacterial suspension was spread onto nutrient agar plates and the extracts were tested for antibacterial activity using 5 mm sterilised filter paper discs. Discs were infused with 10 µL of *Syzygium* spp. extracts, allowed to dry and placed onto the inoculated plates. The plates were left to stand at 4 °C for 2 h before incubation. Plates inoculated with *S. putrefaciens* or *S. loihica* cultures were incubated at 30 °C for 24 h. *S. baltica* or *S. frigidimarina* cultures were incubated at 15 °C for 72 h. The diameters of the inhibition zones

were measured to the closest whole millimetre. Each assay was completed in at least triplicate. Mean values ($\pm$ SEM) are reported in this study. Ampicillin discs (10 µg) were obtained from Oxoid Ltd., Australia and used as positive controls to compare antibacterial activity. Filter discs infused with 10 µL of distilled water were used as a negative control.

Minimum inhibitory concentration (MIC) determination

The minimum inhibitory concentration for each extract was determined using two methods. A liquid dilution MIC assay was employed as it is generally considered the most sensitive bacterial growth inhibitory assay (Eloff 1998). Furthermore, as microplate liquid dilution MIC assays are perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method allows for comparisons with other studies. A solid phase agar disc diffusion assay was also used in this study as this bioassay was deemed to provide a closer representation of the environment and conditions relevant to a solid fish preservative system.

Microplate liquid dilution MIC assay

The MICs of the extracts were evaluated by standard methods (Eloff 1998). Briefly, overnight bacterial cultures were added dropwise to fresh nutrient broth and the turbidity was visually adjusted to produce a McFarlands number 1 standard culture. This was subsequently diluted 1 in 50 with nutrient broth, resulting in the MIC assay inoculum culture. A volume of 100 µL sterile broth was added to all wells of a 96 well plate. Test extracts or control antibiotics (100 µL) were then added to the top row of each plate and 1 in 2 serial dilutions were prepared in each column of wells by transferring 100 µL from the top well to the next well in each column, etc. A growth control (without extract) and a sterile control (without inoculum) were included on each plate. A volume of 100 µL of bacterial culture inoculum was added to all wells except the sterile control wells. Plates inoculated with *S. putrefaciens* or *S. loihica* cultures were incubated at 30 °C for 24 h. Plates inoculated with *S. baltica* or *S. frigidimarina* cultures were incubated at 15 °C for 72 h. p-Iodonitrotrazolium violet (INT) was obtained from Sigma, Australia and dissolved in sterile deionised water to prepare a 0.2 mg/mL INT solution. A 40 µL volume of this solution was added into all wells and the plates were incubated for a further 6 h at 30 °C. Following incubation, the MIC was visually determined as the lowest dose at which colour development was inhibited.

Disc diffusion MIC assay

The minimum inhibitory concentrations (MIC) of the extracts was also evaluated by disc diffusion assay as previously described (Kalt and Cock 2014). Briefly, the plant extracts were diluted in deionised water and tested across a range of concentrations. Discs were impregnated with 10 µL of the extract dilutions, allowed to dry and placed onto inoculated plates. The assay was achieved as outlined above and graphs of the zone of inhibition versus concentration were plotted. Determination of MIC values were achieved using linear regression.

Toxicity screening

Reference toxin for toxicity screening

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared in deionised water (4 mg/mL) and serially diluted in artificial seawater for use in the *Artemia franciscana* nauplii bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was assessed using a modified *Artemia franciscana* nauplii lethality assay (Cock and Ruebhart 2009). Briefly, 400 µL of seawater containing ~46 (mean 45.8, $n = 125$, SD 13.6) *A. franciscana* nauplii were added to wells of a 48 well plate and immediately used in the bioassay. A volume of 400 µL of the reference toxin or the diluted plant extracts were transferred to the wells and incubated at 25 ± 1 °C under artificial light (1000 Lux). For each plate, a 400 µL seawater negative control was run in triplicate. The wells were assessed at regular intervals and the number of dead counted. The nauplii were deemed dead if no movement of the appendages was observed within 10 s. After 24 h, all nauplii were sacrificed and counted to determine the total % mortality per well. The LC_{50} with 95% confidence limits for each treatment was calculated using probit analysis.

Statistical analysis

Data is expressed as the mean $\pm$ SEM of at least three independent experiments.

Results

Liquid extraction yields and qualitative phytochemical screening

Extractions of the *Syzygium* spp. plant materials (1 g) with solvents of varying polarity yielded dried plant extracts

ranging from 62 mg (*S. luehmannii* leaf ethyl acetate extract) to 560 mg (*S. luehmannii* fruit methanolic extract) (Table 1). Ethyl acetate extracts yielded relatively low masses of extracted material compared to the methanolic extracts, which gave much greater yields. The dried extracts were resuspended in 10 mL of deionised water (containing 1% DMSO), resulting in the concentrations presented in Table 1.

Antioxidant content

Antioxidant capacity for the plant extracts (Table 1) ranged from 1.5 (*S. luehmannii* fruit ethyl acetate extract) to 94.6 mg ascorbic acid equivalents per gram of dried plant material extracted (*S. luehmannii* fruit methanolic extract). The antioxidant capacities for the methanolic and aqueous extracts of each plant part were generally similar for both species and were significantly higher than the corresponding ethyl acetate extracts.

Growth inhibition of *Shewanella* spp.

To determine the ability of the *Syzygium* spp. extracts to inhibit *Shewanella* spp. growth, 10 µL of each extract was screened using a disc diffusion assay. *S. putrefaciens* growth was inhibited by 11 of the 12 extracts screened (92%) (Fig. 1a). Only the aqueous *S. luehmannii* leaf was devoid of *S. putrefaciens* growth inhibitory activity. All other *Syzygium* spp. extracts were good inhibitors of *S. putrefaciens* growth, with zones of inhibition generally >7 mm. In general, the *S. australe* extracts were more potent growth inhibitors than the corresponding *S. luehmannii* extracts. Interestingly, whilst the *S. luehmannii* fruit and *S. australe* fruit and leaf aqueous extracts inhibited *S. putrefaciens* growth, they were generally less potent than the corresponding methanol and ethyl acetate extracts. The methanolic *S. australe* fruit and leaf extracts were particularly potent growth inhibitors (as assessed by zone of inhibition), with inhibition zones of 9.0 ± 0.5 and 9.3 ± 0.3 mm respectively. This compares favourably with the ampicillin control (10 µg) which had zones of inhibition of 7.6 ± 0.9 mm. As *S. putrefaciens* is a main causative agent for microbial fish spoilage (at both mesophilic and psychrophilic conditions), this is a noteworthy result.

As seafood is generally stored using low temperature conditions, other psychrotrophic and psychrophilic *Shewanella* spp. have increased importance at lower temperatures (Vogel et al. 2005). Control of *S. baltica* growth, and to a lesser extent *S. frigidimarina* growth, become more important when fish are stored at lower temperatures for extended periods and the contribution of *S. putrefaciens* decreases (Vogel et al. 2005). The growth of *S. baltica* was also susceptible to the *Syzygium* spp. extracts (Fig. 1b).

Table 1 The mass of dried extracted material, the concentration after resuspension in deionised water, qualitative phytochemical screenings and antioxidant capacities (mg ascorbic acid equivalents per g dried plant material) of *Syzygium* spp. extracts

	<i>S. australe</i> fruit				<i>S. australe</i> leaf				<i>S. luehmamii</i> fruit				<i>S. luehmamii</i> leaf			
	Aqueous	Methanolic	Ethyl acetate		Aqueous	Methanolic	Ethyl acetate		Aqueous	Methanolic	Ethyl acetate		Aqueous	Methanolic	Ethyl acetate	
Mass of dried extract (mg)	240	360	110		180	360	110		120	560	130		88	190	62	
Concentration of resuspended extract (mg/mL)	24	36	11		18	36	11		12	56	13		9	19	6	
Total phenolics	+++	+++	++		+++	+++	++		+++	+++	++		+++	+++	++	
Water soluble phenolics	+++	+++	–		+++	+++	–		+++	+++	–		+++	+++	–	
Water insoluble phenolics	++	+++	++		++	+++	++		++	+++	++		++	+++	++	
Cardiac glycosides	–	–	–		–	–	–		–	–	–		–	–	–	
Saponins	+	–	–		+	+	–		+	+	–		+	+	–	
Triterpenes	–	–	–		+	+	–		+	+	–		+	+	–	
Phytosterols	–	–	–		–	–	–		–	–	–		–	–	–	
Meyer test	–	–	–		–	–	–		–	–	–		–	–	–	
Wagner test	–	–	–		–	–	–		–	–	–		–	–	–	
Flavonoids	+++	+++	+		++	+++	++		+++	+++	+		++	+++	++	
Tannins	+	+	–		+	+	–		+	+	–		+	+	–	
Free anthraquinones	–	–	–		–	–	–		–	–	–		–	–	–	
Combined anthraquinones	–	–	–		–	–	–		–	–	–		–	–	–	
Antioxidant content by DPPH reduction ^a	40.7	55.2	9.2		25.3	40.2	2.58		59.2	94.6	1.5		44.7	43.4	5.5	

+++ indicates a large response, ++ indicates a moderate response, + indicates a minor response, – indicates no response in the assay

^a expressed as mg ascorbic acid equivalents per 1 g plant material extracted

Fig. 1 Growth inhibitory activity of *S. australe* and *S. leuhammii* fruit and leaf extracts against the psychrotrophic **a** *S. putrefaciens* and **b** *S. baltica* environmental isolates measured as zones of inhibition (mm). Results are expressed as mean zones of inhibition $\pm$ SEM

Consistent with the trend noted for *S. putrefaciens* growth inhibition, *S. baltica* also appeared more susceptible to the methanolic extracts than to the corresponding aqueous and ethyl acetate extracts. The methanolic *S. australe* leaf and *S. leuhammii* leaf ethyl acetate extracts were particularly potent inhibitors of *S. baltica* growth, with zones of inhibition >12.3 mm. Furthermore, the inhibition of *S. baltica*

growth was particularly noteworthy as the growth of this bacterium was unaffected by ampicillin, indicating that this is an antibiotic resistant strain. Several other *Syzygium* spp. extracts were also good inhibitors of *S. baltica* growth (as determined by zones of inhibition). Aside from the increased inhibition of the methanolic *S. australe* leaf extract compared to its *S. leuhammii* counterpart, no

obvious differences were noted for the growth inhibitory efficacy between the *Syzygium* spp.

Growth of *S. frigidimarina* was also strongly inhibited by the *Syzygium* spp. extracts (Fig. 2a). Indeed, only the aqueous and ethyl acetate *S. leuhammii* leaf extracts were completely devoid of *S. frigidimarina* growth inhibitory activity. As evident for *S. putrefaciens* and *S. baltica* growth inhibition, the methanolic extracts were more potent bacterial growth inhibitors than were the corresponding aqueous and ethyl acetate extracts. The methanolic *S. australe* fruit extract was a particularly growth inhibitor, with an inhibition zone of 10.6 ± 1.0 mm. In general, the *S. australe* extracts were more potent growth inhibitors than the corresponding *S. leuhammii* extracts (as determined by zones of inhibition). Furthermore, the fruit extracts were moderately more potent *S. frigidimarina* growth inhibitors than the corresponding leaf extracts. As seen for the other psychrotrophic bacterial species (*S. baltica*), *S. frigidimarina* growth was also resistant to ampicillin exposure.

S. loihica and *S. putrefaciens* share similar genotypic and phenotypic characteristics and have similar optimal growth conditions (Gao et al. 2006). Thus, the ability of the *S. australe* and *S. leuhammii* fruit and leaf extracts to inhibit *S. loihica* was also tested (Fig. 2b). In contrast with the other *Shewanella* spp., *S. loihica* was particularly susceptible to the ampicillin control (zone of inhibition of 21 ± 3.4 mm). Whilst substantially smaller zones of inhibition were recorded against the *Syzygium* extracts, several displayed potent *S. loihica* growth inhibition. Indeed, exposure of *S. loihica* to the methanolic and aqueous *S. australe* fruit extracts, the methanolic *S. australe* leaf extract and the methanolic *S. leuhammii* fruit extract all produced >10 mm zones of inhibition. Interestingly, a substantial difference was noted between the inhibition by the different *Syzygium* spp. With the exception of the leaf ethyl acetate extract, all *S. australe* extracts inhibited *S. loihica* growth. In contrast, only the methanolic *S. leuhammii* extracts inhibited growth.

Quantification of minimum inhibitory concentration (MIC)

The relative level of antimicrobial activity was further evaluated by determining the MIC values (Table 2) for each extract against the *Shewanella* spp. which were shown to be susceptible in the disc diffusion screening assays. A similar trend was noted as seen for the screening assays i.e. the *S. australe* extracts were generally better inhibitors of all *Shewanella* spp. growth than were the corresponding *S. leuhammii* extracts. Furthermore, with some notable exceptions, the methanolic extracts were generally the most potent growth inhibitors. The methanolic *S. australe* fruit

extract was a particularly potent *S. putrefaciens* growth inhibitor, with disc diffusion (DD) and liquid dilution (LD) MIC values of 640 and 600 µg/mL respectively. That extract was also a potent inhibitor of *S. baltica* (DD MIC 162 µg/mL; LD MIC 150 µg/mL), *S. frigidimarina* (DD MIC 269 µg/mL; LD MIC 300 µg/mL) and *S. loihica* growth (DD MIC 407 µg/mL; LD MIC 87 µg/mL). The aqueous and ethyl acetate *S. australe* fruit extracts also had low MIC values against all *Shewanella* spp., (generally ≤ 800 µg/mL against all *Shewanella* spp. except *S. frigidimarina*), also indicating their potential as fish preservatives.

The *S. australe* leaf extracts were also potent growth inhibitors of *Shewanella* spp. growth. The leaf MIC values were generally approximately twice those determined for the fruit extracts. However, particularly potent inhibition was noted for the inhibition of *S. loihica* growth by the aqueous *S. australe* leaf extract (DD MIC values 98 µg/mL; LD MIC 281 µg/mL). Low MIC values indicative of potent to mid potency growth inhibitory activity were also noted for the *S. australe* leaf extracts against the other *Shewanella* spp. In contrast to the potent growth inhibition of the *S. australe* extracts, the *S. leuhammii* extracts were generally less potent growth inhibitors. With the exception of *S. frigidimarina* inhibition by the aqueous *S. leuhammii* extract (DD MIC 371 µg/mL; LD MIC 800 µg/mL) which was a substantially more potent inhibitor of *S. frigidimarina* growth than was the aqueous *S. australe* extract (DD MIC 1237 µg/mL; LD MIC 1500 µg/mL), all other *S. leuhammii* extracts had much higher MIC values than the corresponding *S. australe* extracts.

Quantification of toxicity

All extracts were initially screened in the assay at 2000 µg/mL (Fig. 3). Additionally, potassium dichromate was also tested in the bioassay as a reference toxin. The reference toxin was rapid in its onset of mortality, promoting nauplii death within the first 3 h of exposure, with 100% mortality evident within 5 h (unpublished results). With the exception of ethyl acetate leaf extracts of both species, all extracts showed >50% mortality rates after 24 h. As toxicity of crude plant extracts has previously been defined as 24 h LC₅₀ values <1000 µg/mL (Cock and Ruebhart 2009), the ethyl acetate leaf extracts of both species were deemed to be nontoxic.

To further quantify the effects of toxin concentration on the initiation of mortality, the extracts were serially diluted in artificial seawater to test across a range of concentrations in the *Artemia* nauplii bioassay. The 24 h LC₅₀ values of the *Syzygium* spp. extracts towards *A. franciscana* are displayed in Table 2. No LC₅₀ were detected for the leaf or fruit ethyl acetate extract of either

Fig. 2 Growth inhibitory activity of *S. australe* and *S. leuhamanii* fruit and leaf extracts against the psychrotrophic **a** *S. frigidimarina* and **b** *S. loihica* environmental isolates measured as zones of inhibition (mm). Results are expressed as mean zones of inhibition $\pm$ SEM

species as <50% mortality was seen in all tested concentrations. Extract with LC_{50} values >1000 $\mu\text{g}/\text{mL}$ towards *Artemia* nauplii have been defined as being nontoxic in this assay (Cock and Ruebhart 2009). The *S. australe* aqueous and methanolic fruit extracts both had LC_{50} values >1000 $\mu\text{g}/\text{mL}$ and were thus determined to be non-toxic. Conversely, the aqueous and methanolic

S. australe leaf extracts as well as the *S. leuhamanii* leaf and fruit extracts returned LC_{50} values substantially <1000 $\mu\text{g}/\text{mL}$ and were considered to be toxic. This was surprising as the fruit from both *S. australe* and *S. leuhamanii* are considered nutritious and non-toxic for human consumption. However, the *A. franciscana* assay is sensitive to extreme pH ranges and samples with high

Table 2 Disc diffusion and liquid dilution MICs against *S. putrefaciens*, *S. baltica*, *S. frigidimarina* and *S. loihiica* growth ($\mu\text{g/mL}$) and *Artemia* nauplii bioassay LC_{50} values ($\mu\text{g/mL}$) of *S. australe* and *S. luehmanni* fruit and leaf extracts

<i>Syzygium</i>		<i>S. putrefaciens</i>		<i>S. baltica</i>		<i>S. frigidimarina</i>		<i>S. loihiica</i>		<i>Artemia</i> nauplii bioassay LC_{50}
Plant	Extract	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	DD MIC	LD MIC	
<i>S. australe</i>	Fruit/aqueous	803	800	344	400	1237	1500	875	375	3310
	Fruit/methanolic	640	600	162	150	269	300	407	86	1879
	Fruit/ethyl acetate	903	700	690	700	2300	700	1000	344	—
	Leaf/aqueous	2104	2300	443	600	2460	1100	98	281	244
	Leaf/methanolic	586	1100	271	1100	198	600	453	563	294
	Leaf/ethyl acetate	1635	2800	1220	1400	1300	1400	—	683	—
<i>S. luehmanni</i>	Fruit/aqueous	1415	1500	—	1500	371	800	—	750	478
	Fruit/methanolic	>10,000	1800	>10,000	1800	>10,000	3500	>10,000	1750	414
	Fruit/ethyl acetate	2303	3300	2700	1600	2440	1600	—	1600	—
	Leaf/aqueous	—	2800	2042	2300	—	2300	—	1125	813
	Leaf/methanolic	3083	4800	3606	4800	1265	2400	2769	593	450
	Leaf/ethyl acetate	4000	6000	2770	6000	—	—	—	—	—

Numbers indicate the mean DD MIC, LD MIC and LC_{50} values of triplicate determinations, — indicates no inhibition
DD disc diffusion, *LD* liquid dilution

Fig. 3 The lethality of the *S. australe* and *S. luehmanni* fruit and leaf extracts (2000 $\mu\text{g/mL}$) and the potassium dichromate control (1000 $\mu\text{g/mL}$) towards *Artemia franciscana* nauplii after 24 h exposure. Results are expressed as mean % mortality $\pm$ SEM

ascorbic acid content may adversely alter the pH. Of note, all of the extracts that returned LC_{50} values $<1000 \mu\text{g/mL}$ in our study had relatively high antioxidant capacities (Table 1). Furthermore, previous studies (Jamieson et al. 2014) reported that the high antioxidant capacities of

these *Syzygium* spp. is predominately due to their high ascorbic acid contents. Thus, it is probable that the apparent toxicity reported in this study is a fallacious result due to the high ascorbic acid content of the extracts and these are in fact non-toxic.

Discussion

Low temperature storage is currently the main method of preserving fresh fish and other seafood. Whilst this is an effective method of controlling the growth of many food spoilage bacteria, it is inefficient at inhibiting the growth of psychrophilic and psychrotrophic bacteria such as the *Shewanella* spp. and other preservation methods are required. Decreasing the water activity by drying the fish and/or by adding salt, or alteration of the pH of the fish muscle by fermenting fish or directly adding acids (e.g. acetic, citric, lactic) are effective at inhibiting bacterial growth in stored fish. However, these methods also have profound effects on the taste and textural characteristics of the fish. Furthermore, health concerns associated with excess sodium consumption has resulted in a decreased use of salt as a preservative in recent years. Other methods of delaying fish spoilage entail the addition of chemical preservatives. Commonly used chemical food preservatives include butylhydroxyanisol (BHA), butylated hydroxytoluene (BHT), nitrates, nitrites, sulfur dioxide (SO₂) and sulfites (SO₃) (Ray and Bhunia 2008). Of concern, the safety of many of the chemical preservatives used in food has yet to be determined and in some cases these preservatives have been linked with serious health problems. Studies have indicated that chemical preservatives may cause respiratory problems (Simon 2004), aggravate attention deficit hyperactivity disorder (ADHD) and cause anaphylactic shock in susceptible individuals (Simon 2004). Due to greater consumer awareness and the negative perceptions of artificial preservatives, consumers are increasingly avoiding foods containing preservatives of chemical origin. Natural antimicrobial alternatives are increasingly being sought to increase the shelf life and safety of processed foods.

S. australe and *S. leuhammannii* were selected for screening for the ability to block the growth of fish spoilage bacteria as they have potential to positively influence the shelf life of fish in several ways. A major portion of fresh fish spoilage is the result of oxidative spoilage. The treatment of fish with preparations containing high antioxidant contents (e.g. some plant extracts) decreases lipid oxidation and thus inhibits oxidative rancidity (Pazos et al. 2005). *S. australe* and *S. leuhammannii* fruit have previously been reported high to have very high antioxidant capacities (Jamieson et al. 2014) and thus have potential in reducing oxidative rancidity. Indeed, Trolox equivalency antioxidant content (TEAC) values of approximately 27 and 28 µmol TE/g of extracted fruit have previously been reported for *S. australe* and *S. leuhammannii* methanolic extracts respectively (compared to 39 µmol TE/g for blueberries). The same study also reported ascorbic acid levels of 0.7 and

1.8 µmol/g extracted fruit for the *S. australe* and *S. leuhammannii* extracts respectively (compared to 0.08 µmol ascorbic acid/g blueberries). Our study also reports high antioxidant capacities for the *S. australe* and *S. leuhammannii* extracts using an alternative measure of antioxidant capacity (DPPH free radical scavenging assay). Using this assay, we measured antioxidant capacities to be as high as 94 mg ascorbic acid per g of dried extracted plant material (methanolic *S. leuhammannii* fruit extract). This is an exceptionally high antioxidant capacity (equating to 9.4% of the original dry plant material) and would be expected to provide a high degree of protection against oxidative fish spoilage. The contribution of antioxidant protection to food spoilage has been extensively reported elsewhere (Pazos et al. 2005) and was not therefore a focus of our study.

The other criterion for our selection *S. australe* and *S. leuhammannii* as potential fish preservatives was their ability to inhibit the growth of other bacteria. Previous studies have reported antibacterial activity for *S. australe* and *S. leuhammannii* fruit (Sautron and Cock 2014) and leaf extracts (Cock 2012) against a wide variety of pathogenic and food spoilage bacteria. Furthermore, potent growth inhibitory activity has also been reported for other Australian *Syzygium* spp. (Chikowe et al. 2013), as well as Asian (Mohanty and Cock 2010) and African species (Cock and van Vuuren 2015). Despite this, no previous studies have examined the ability of *S. australe* and *S. leuhammannii* to inhibit *Shewanella* spp. growth.

Our study has confirmed the potential of *S. australe* and *S. leuhammannii* extracts for delaying fish spoilage and increasing seafood shelf life. Both *Syzygium* spp. displayed considerable growth inhibitory activity against all *Shewanella* spp. tested. The methanolic *S. australe* fruit extract was particularly promising, with MIC values of approximately 600, 150, 300 and 90 µg/mL against *S. putrefaciens*, *S. baltica*, *S. frigidimarina* and *S. loihica* respectively. Studies using extracts from other plants have reported comparable or considerably higher MIC values as signifying potent *Shewanella* spp. growth inhibitory activity. One recent study reported an MIC value of 512 µg/mL against a different environmental *S. putrefaciens* isolate by an ethanolic *Zataria multiflora* extract (Motevasel et al. 2011). *Zataria multiflora* is commonly used in the Middle East as both a natural food preservative, and as a medicinal plant. Based on its antiseptic properties, it is considered to have potent growth inhibitory properties against a wide variety of pathogenic and non-pathogenic bacteria (Motevasel et al. 2011). A different study reported moderate growth inhibition (2 mg/mL) of *S. putrefaciens* by aqueous *Terminalia catappa* extracts (Chansue and Assawawongkasem 2008). This plant is widely regarded for its antibacterial properties. Thus, the significantly

greater potency reported in our study emphasises the efficacy of the *S. australe* and *S. leuhamnii* extracts. However, it is noteworthy that the *T. catappa* study used a different MIC assay than either of the assays used in our study and this may account for the relatively high MIC value reported in that study. Whilst the earlier studies examined *S. putrefaciens* growth inhibition, neither screened against the other *Shewanella* spp. tested in our study, so these comparisons are not available.

Although the methanolic *S. australe* fruit extract was the most promising extract in our studies, all of the other *S. australe* and some of the *S. leuhamnii* extracts were also potent inhibitors of *Shewanella* spp. growth, albeit with higher MIC values. This indicates that they also have potential as fish preservatives. The aqueous *S. australe* fruit extracts for example had MIC values only marginally higher than determined for the methanolic extract, indicating their similar potency. Given their similar efficacy, the aqueous *S. australe* fruit extract may be a better option as a food preservative. The methanol and ethyl acetate solvents were thoroughly evaporated following extraction and the extracted material redissolved in deionised water. However, as organic solvents were initially used in preparing these extracts, consumers may mistakenly believe that these extracts contain these solvents. Whilst this is not so, the aqueous extracts do not suffer from the same misconceptions, and given their similar potency, may be more acceptable for food preservation.

Considerably fewer studies have examined the inhibitory properties of plant extract against the other *Shewanella* spp. examined in our study. However, an interesting trend was noted in the literature: Several authors reported antibiotic resistance in multiple *Shewanella* spp. (Poirel et al. 2005). In particular, many strains were highly resistant to β -lactam antibiotics. Similarly, both of the psychrotrophic *Shewanella* spp. examined in our study (*S. baltica*, *S. frigidimarina*) were completely unaffected by relatively high doses of ampicillin. Interestingly, *S. baltica*, *S. frigidimarina* were both highly susceptible to the *Syzygium* spp. extracts, indicating that the bioactive compounds in these extracts do not have β -lactam structures and/or function via mechanisms different to the conventional β -lactam antibiotics. This has interesting implications beyond the use of *S. australe* and *S. leuhamnii* extracts as natural fish preservatives. Indeed, these extracts may have further potential as antibiotics against β -lactam resistant pathogens. Further studies are currently underway in our laboratory, both to screen these extracts against multiple bacterial species/strains, and to determine their antibiotic mechanism(s).

Two different MIC assays were chosen to quantify the *Shewanella* spp. growth inhibition potency in our studies. As fish is a solid medium, solid phase MIC techniques were

considered the best to represent the environment in a fish preservation model. Thus, a modified disc diffusion assay was used to approximate the solid environment of a fish. A microplate liquid dilution MIC assay was also employed as a number of negative aspects are often associated with disc diffusion assays. Disc diffusion assays are perceived as often yielding false negative results (or artificially low potency), as volatile compounds may evaporate from the surface of the agar. However, this is generally considered to be more problematic when essential oils are used rather than when extracts are tested as in our study. It is also likely that the most volatile components would already be depleted from the plant material during the extensive dehydration protocol, prior to preparation of the extracts and thus it is doubtful that significant further evaporation would occur from the discs.

The diffusion of phytochemicals through an agar gel is also influenced by other factors which may affect the determination of the apparent MIC:

- As disc diffusion methods are reliant on diffusion of molecules through the aqueous environment of an agar gel, the MIC may be affected by the solubility of the extract compounds in the aqueous environment. Polar compounds easily diffuse within the gel, whereas less soluble compounds do not diffuse as readily and thus are concentrated around the disc. Therefore, disc diffusion assays are often thought to provide falsely low MICs when nonpolar compounds are responsible for the growth inhibition.
- Diffusion of molecules within a gel is also affected by the size of those molecules. Thus the diffusion of large, complex phytochemicals (e.g. complex tannins) within agar gels would also be retarded and may provide a false idea of the efficacy of an extract.

Due to these perceived short comings for the disc diffusion assay, we also used a microplate liquid dilution MIC assay. Furthermore, it is generally believed that this assay is substantially more sensitive than the disc diffusion assay (Eloff 1998). However, it is noteworthy that many practitioners of disc diffusion assays use semi-quantitative analysis and determine the MIC as the highest dilution of sample still able to inhibit bacterial growth. In our study, we utilised a modified, quantitative approach which measured the size of the inhibition zone as a function of concentration and used linear regression to extrapolate and get a more accurate determination of the MIC. Using this modified assay, we achieved a good correlation between MIC values obtained from both protocols. Indeed, examples such as the disc diffusion (DD) and liquid dilution (LD) MIC values for the inhibition of the aqueous (DD MIC 803 $\mu\text{g/mL}$; LD MIC 800 $\mu\text{g/mL}$), methanolic (DD MIC 640 $\mu\text{g/mL}$; LD MIC 600 $\mu\text{g/mL}$), and ethyl acetate

(DD MIC 903 µg/mL; LD MIC 700 µg/mL) *S. australe* fruit extracts against *S. putrefaciens* demonstrate the close correlation of these MIC values. As microplate liquid dilution MIC assays are now perhaps the most commonly used method of quantifying bacterial growth inhibition efficacy, use of this method also allows for direct comparisons with other studies.

Whilst a detailed investigation of the phytochemistry of the *Syzygium* spp. extracts tested was beyond the scope of our study, the qualitative phytochemical studies highlighted several classes of compounds which may contribute to their bacterial growth inhibitory properties. Flavonoids were particularly prevalent in all extracts. Many studies have reported potent antibacterial activities for a wide variety of flavonoids (Narayana et al. 2001). Tannins were also present in most extracts, albeit in relatively lower levels. Multiple tannins have been reported to inhibit the growth of a broad spectrum of bacterial species (Buzzini et al. 2008) through a variety of intra- and extracellular mechanisms (Buzzini et al. 2008; Wolinsky and Sote 1984; Hogg and Embery 1982; Wu-Yuan et al. 1988). It is likely that other phytochemical classes may also contribute to the growth inhibitory properties of these extracts. Further phytochemical evaluation studies and bioactivity driven isolation of active components is required to evaluate the mechanism of *Shewanella* spp. growth inhibition.

The findings reported here demonstrate that all *S. australe* fruit extracts were nontoxic towards *Artemia* nauplii and are thus safe to use as natural fish preservatives. Interestingly, these extracts were also the most potent *Shewanella* spp. growth inhibitors, indicating that they have the greatest potential for development as preservatives. In contrast, the *S. australe* leaf extracts and all *S. luehmannii* extracts displayed substantial toxicity, with LC₅₀ values as low as 244 µg/mL (aqueous *S. australe* leaf extract). Extracts with LC₅₀ values ≤1000 µg/mL towards *Artemia* nauplii are defined as being toxic (Cock and Ruebhart 2009). This apparent toxicity may impact on the use of these extracts as fish preservatives. The *Artemia* nauplii toxicity assay was chosen for analysis in our studies as it has been reported to closely correlate with cell line assays, as well as its ease of use and reproducibility (Cock and Ruebhart 2009). However, *Artemia* nauplii are particularly sensitive to pH changes. As the *Syzygium* spp. extracts generally had high ascorbic acid contents, it is likely adverse pH changes may be responsible for the *Artemia* nauplii mortality, and that the apparent toxicity reported in this study is a fallacious result. However, further toxicity studies using human cell lines and subsequent in vivo studies are required to confirm the safety of these extracts before they are accepted as natural fish preservative alternatives.

Conclusions

The results of this study demonstrate that *S. australe* and *S. luehmannii* fruit and leaf extracts are potent inhibitors of *Shewanella* spp. growth and therefore have potential as natural fish/seafood preservatives. The *S. australe* fruit extracts were particularly effective against all psychrotrophic and mesophilic *Shewanella* spp. and thus have potential for both fresh and cold storage fish preservation.

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