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The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*

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J. WAN, A. WILCOCK AND M.J. COVENTRY. 1998. Basil essential oils, including basil sweet linalool (BSL) and basil methyl chavicol (BMC), were screened for antimicrobial activity against a range of Gram-positive and Gram-negative bacteria, yeasts and moulds using an agar well diffusion method. Both essential oils showed antimicrobial activity against most of the micro-organisms examined except *Clostridium sporogenes*, *Flavimonas oryzihabitans*, and three species of *Pseudomonas*. The minimum inhibitory concentration (MIC) of BMC against *Aeromonas hydrophila* and *Pseudomonas fluorescens* in TSYE broth (as determined using an indirect impedance method) was 0·125 and 2% (v/v), respectively; the former was not greatly affected by the increase of challenge inoculum from 10^3 to 10^6 cfu ml^{-1}. Results with resting cells demonstrated that BMC was bactericidal to both *Aer. hydrophila* and *Ps. fluorescens*. The growth of *Aer. hydrophila* in filter-sterilized lettuce extract was completely inhibited by 0·1% (v/v) BMC whereas that of *Ps. fluorescens* was not significantly affected by 1% (v/v) BMC. In addition, the effectiveness of washing fresh lettuce with 0·1 or 1% (v/v) BMC on survival of natural microbial flora was comparable with that effected by 125 ppm chlorine.

INTRODUCTION

Minimally processed fresh (MPF) vegetable products packaged in modified atmospheres are a growing market segment for horticultural produce (Ahvenainen 1996). However, effective refrigerated temperature control during manufacture, distribution and retailing is required for maintaining the microbiological quality and safety of these products. Psychrotrophic pathogens (such as *Aeromonas*, *Listeria*, *Yersinia* and some types of *Clostridium botulinum*) and spoilage bacteria (such as *Pseudomonas* and Enterobacteriaceae) are the major microbiological concerns with MPF products (Lund 1992; Nguyen-the and Carlin 1994; Beuchat 1996). In particular, *Aer. hydrophila* poses a potential risk as it has been reported to occur at high microbial loads in these products (up to 10^4–10^6 cfu g^{-1}, Marchetti *et al.* 1992) and cytotoxicity occurs in a large proportion of *Aeromonas* isolates from various sources (Callister and Agger 1987; Fricker and Tompsett 1989).

Preservation systems using food-grade antimicrobials offer the potential of additional hurdles for more effective control of the microbiological quality of MPF vegetable products throughout the processing and distribution chains. There is also an increasing trend to the development of ready-to-serve salad products and the inclusion of herbs and unusual varieties in MPF vegetable products (Anon 1993; Anon 1996). Sweet basil (*Ocimum basilicum* L.) is a popular culinary herb and has been used widely as a food ingredient for flavouring confectionary and baked goods, condiments (tomato pastes, sauces, pickles and vinegars) and meat products such as sausages and canned meats (Dziezak 1989). In addition, basil essential oils have been reported to be antimicrobial against a variety of Gram-positive, Gram-negative bacteria and yeasts and moulds (Reuveni *et al.* 1984; Deans and Ritchie 1987; Dube *et al.* 1989; Gangrade *et al.* 1989; Meena and Sethi 1994). It has also been demonstrated that the antimicrobial activities of essential oils of different species of *Ocimum* were predominantly associated with the main constituents of linalool and methyl chavicol (Sinha and Gulati 1990). In the current study, the effects of commercially available basil essential oils were screened against a variety of bacteria, yeasts and moulds occurring in foods to verify the spectrum of activity. Antimicrobial activity against *Aer. hydrophila* and *Ps. fluorescens* was further characterized with resting cells and growing cells in broth medium and filter-sterilized lettuce extract. In addition, the effect of washing with basil oil suspensions on survival of the natural microflora of fresh lettuce

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was determined and compared with a chlorine (added as hypochlorite) wash.

MATERIALS AND METHODS

Cultures, media and essential oils

Cultures used in this investigation were all obtained from the culture collection of the Australian Food Industry Science Centre (AFISC), Werribee, Victoria, Australia, and media used were all obtained from Oxoid, P/L, West Heidelberg, Victoria, Australia. Stock cultures were maintained at –80 °C in Nutrient broth containing 16% (v/v) glycerol. Bacterial cultures were propagated as described in Coventry *et al.* (1996). In addition, *Flavimonas* and *Yersinia* were propagated for 16 h in Tryptone Soy Broth with added 0·6% yeast extract (TSYE) at 30 °C. Yeasts were grown in Oxytetracycline Glucose Yeast Extract (OGY) broth for 16 h at 25 °C. Moulds were grown on the surface of OGY agar for 5 d at 25 °C, and spore suspensions of mould cultures were prepared by the method of Batish *et al.* (1989) and held at 4 °C. Basil essential oils, including basil sweet linalool (BSL) and basil methyl chavicol (BMC), were obtained from Auroma P/L, Hallam, Victoria, Australia.

Agar well diffusion method

Freshly grown bacterial and yeast cultures and mould spore suspensions were inoculated (10^6 cfu ml^{-1} for bacteria and yeasts and 10^5 spores ml^{-1} for moulds) in the appropriate agar medium (15 ml in Petri dishes) in triplicate and wells (4 mm diameter) were made with a sterile cork borer. Essential oils (10 μl) were added to the wells and the same volume of sterile paraffin oil was used as a control. The plates were incubated at the temperature required for each culture, and the radius of the zone of growth inhibition around the wells was measured. Results were quoted after subtracting the radius of the well.

Determination of MIC of basil essential oil against *Aeromonas hydrophila* and *Pseudomonas fluorescens* by indirect impedance

Basil essential oil was dispersed in TSYE broth at 2·0% (v/v) by vortexing at room temperature for 1 min. Twofold serial dilutions were immediately prepared in TSYE to obtain concentrations of 1·0, 0·5, 0·25, 0·125, 0·063, 0·062, 0·016 and 0·008% (v/v) of essential oil. The resultant suspensions were inoculated with 10^3 or 10^6 cfu ml^{-1} *Aer. hydrophila* (AFISC 0110) or *Ps. fluorescens* (AFISC 3105) and incubated at 30 °C in an indirect impedance test system (Owens *et al.* 1989; Bolton 1990) utilizing a RABIT impedance instrument (Don Whitley Scientific Limited, Shipley, UK). Conductivity changes were recorded at 6 min intervals for 48 h. The time to detection of growth (TTD) was indicated by three consecutive changes in conductivity greater than 10 μS/6 min (detection criterion value based on manufacturer's recommendation and the noise level of the conductivity variation of a control TSYE broth). The minimum inhibitory concentration (MIC) was assessed as the lowest concentration of essential oil required for complete inhibition of the test organism after 48 h incubation.

Effect of basil essential oil on resting cells of *Aer. hydrophila* and *Ps. fluorescens* in saline

Cells of *Aer. hydrophila* and *Ps. fluorescens* were harvested from overnight cultures by centrifugation (10 000 *g*, 10 °C, 5 min) and resuspended in sterile saline (0·9% NaCl) to obtain a cell concentration of 10^5 cfu ml^{-1}. Essential oil was added to portions of the cell suspension to 0·1 and 1·0% (v/v). After incubation at 20 °C for 10 min, the cells (from a 1·0 ml sample) were collected by centrifugation (10 000 *g*, 10 °C, 5 min), washed with 1 ml of 0·1% peptone and resuspended in 1·0 ml of 0·1% peptone for viable count determination on TSYE agar.

Effect of basil essential oil on growth of *Aer. hydrophila* and *Ps. fluorescens* in fresh lettuce extract

Fresh lettuce (Iceberg Green obtained from a local supermarket) was rinsed with tap water, drained and homogenized in an equal weight of distilled water using an electric blender. The resultant lettuce homogenate was centrifuged (10 000 *g*, 10 °C, 20 min) and the supernatant filter sterilized (0·45 μm). Basil methyl chavicol was dispersed in the lettuce supernatant at 0·1 and 1% (v/v) and volumes (20 ml) of the resultant medium were inoculated with 10^5 cfu ml^{-1} of *Aer. hydrophila* or *Ps. fluorescens*. After incubation at 10 °C for the time intervals indicated (up to 14 d), aliquots (1 ml) were removed for viable count determination on TSYE agar.

Effect of washing with basil essential oil on survival of natural flora in fresh lettuce

Leaves from three fresh lettuces (Iceberg Green obtained from a local supermarket) were cut into approximately 3 × 3 cm square pieces, pooled, and divided into 25 g portions. Duplicate portions were homogenized in 225 ml of 0·1% peptone, serially diluted and plated onto Plate Count Agar (PCA), *Pseudomonas* agar with CFC supplement (CFC), *Aeromonas* agar with ampicillin supplement (AA) and Violet Red Bile Glucose Agar (VRBGA) for enumeration of total

viable count and presumptive *Pseudomonas*, *Aeromonas* and Enterobacteriaceae, respectively. Other portions (25 g) of lettuce were immersed in 225 ml of either BMC suspension (0·1 and 1%, v/v, dispersed in water), 125 ppm chlorine (delivered as 0·026% v/v hypochlorite solution, Ajax, Australia) or sterile distilled water and gently shaken three times during a 10 min period at 20 °C. The washing liquid was then drained and the lettuce was rinsed with 225 ml sterile distilled water prior to stomaching in 225 ml 0·1% peptone for viable count determinations on PCA, CFC, AA and VRBGA.

RESULTS

Effect of essential oils on growth of micro-organisms on agar media

A wide range of micro-organisms including Gram-positive (11) and Gram-negative (13) bacteria, yeasts (8) and moulds (3) were examined against BMC and BSL using a well diffusion method (Table 1). Both essential oils showed antimicrobial activities against most (25 out of 35 strains) of the micro-organisms examined, demonstrating distinct zones of

Table 1 Effect of basil oil on growth of micro-organisms using an agar well diffusion method

Micro-organisms		Radius (mm) of zone of inhibition by basil essential oil	
AFISC No.	Culture	BSL	BMC
0110	*Aeromonas hydrophila*	3	3
0114	*Aer. hydrophila*	3	3
0303	*Bacillus cereus*	5	4
0320	*B. subtilus*	4	3
0605	*Brochothrix thermosphacta*	1	2
0914	*Clostridium sporogenes*	0	0
1105	*Debaryomyces hansenii*	2	1
1201	*Enterobacter aerogenes* NCTC 10006	1	1
1204	*Ent. agglomerans*	1	1
1301	*Escherichia coli* NCTC 8196	4	3
1406	*Flavimonas oryzihabitans*	0	0
2102	*Lactobacillus plantarum*	2	2
2103	*Lact. curvatus*	2	2
2206	*Leuconostoc cremoris*	3	2
2305	*Listeria innocua*	1	2
2310	*L. monocytogenes*	2	2
2612	*Mucor piriformis*	>13	>13
2802	*Penicillium candidum*	1	8
2804	*P. expansium*	>13	>13
3020	*Proteus vulgaris*	4	3
3101	*Pseudomonoas aeruginosa*	0	0
3105	*Ps. fluorescens*	0	0
3108	*Ps. putida*	0	0
3301	*Saccharomyces cerevisiae*	4	4
3412	*Salmonella typhimurium*	5	4
3501	*Serratia marcescens*	3	2
3601	*Staphylococcus aureus* NCTC 6571	4	3
3801	*Yersinia enterocolitica*	3	2
3901	*Enterococcus faecalis*	1	1
4202	*Candida colliculosa*	4	4
4206	*C. formata*	2	4
4208	*C. humicola*	5	4
4304	*Cryptococcus laurentii*	5	3
4704	*Rhodotorula* sp.	4	2
5001	*Zygosaccharomyces bailii*	5	4

growth inhibition with radii of 1–5 mm (from the edge of the sample wells). *Penicillium expansium* and *Mucor piriformis* showed the greatest sensitivity to both essential oils with the radius of the zone of growth inhibition greater than 13 mm. On the other hand, *Clostridium sporogenes*, *Flavimonas oryzihabitans* and all the three species of *Pseudomonas* tested did not show any sensitivity to either BMC or BSL. Paraffin oil control did not produce any detectable zone of growth inhibition in any of the indicator culture lawns.

MIC of basil essential oils against *Aeromonas hydrophila* and *Pseudomonas fluorescens*

The inhibitory effect (expressed as the time for the detection of growth by indirect impedance) of BSL and BMC on growth of *Aer. hydrophila* (AFISC 0110) and *Ps. fluorescens* (AFISC 3105) (both inoculated at 10^3 or 10^6 cfu ml^{-1}) was determined in TSYE broth at 30 °C (Fig. 1). The addition of 0·063% (v/v) BMC to TSYE broth delayed the growth of

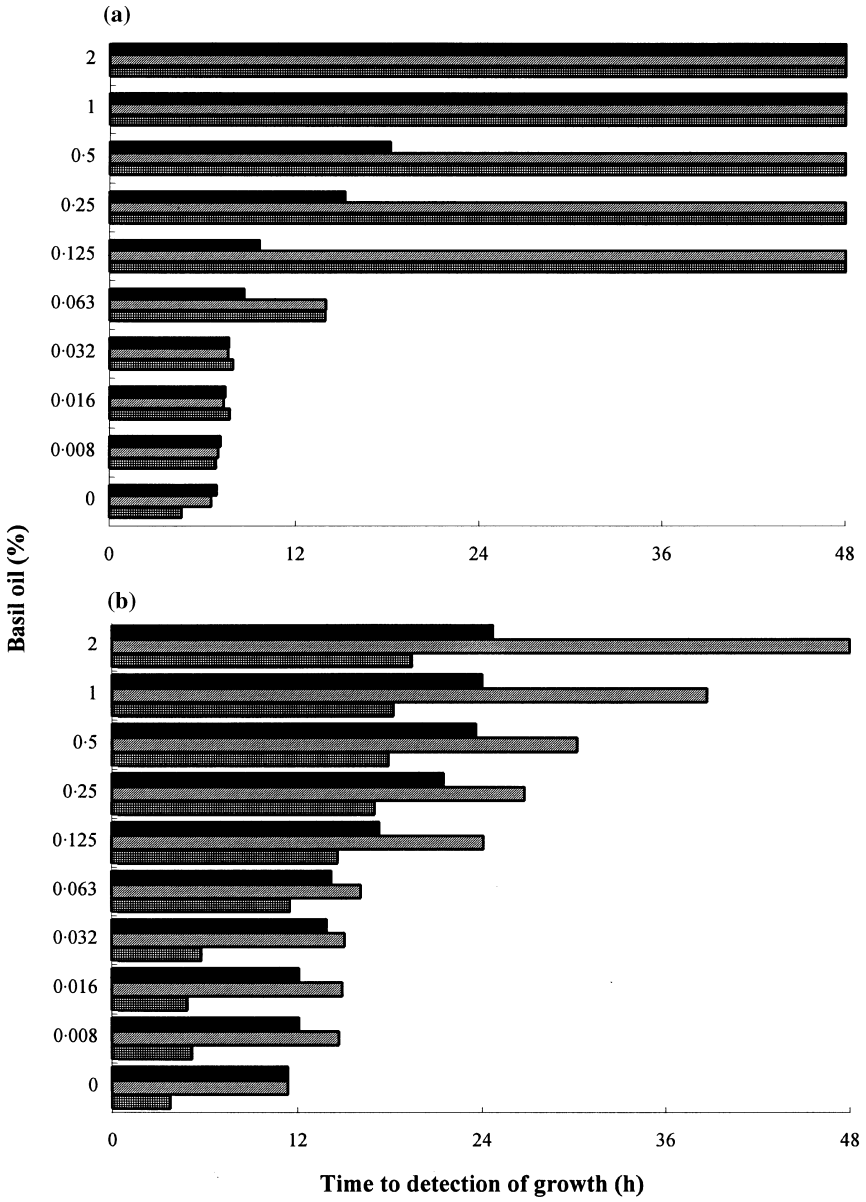

Fig. 1 Effect of concentration of BSL (■) and BMC (▨, ▤) on time to detection of growth of (a) *Aeromonas hydrophila* and (b) *Pseudomonas fluorescens* at inoculum levels of 10^3 (■, ▨) and 10^6 cfu ml^{-1} (▤) in TSYE broth at 30 °C using an indirect impedance method

Aer. hydrophila by up to 7 h and addition of BMC at an amount greater than 0·125% (v/v) (MIC) completely inhibited the growth for the period of the test (48 h) (Fig. 1a). Basil sweet linalool was less antimicrobial against *Aer. hydrophila* than BMC with a higher MIC (1%, v/v). Subsequent plating out of the MIC tube (1 ml sample) in TSYE agar revealed no viable cells of *Aer. hydrophila*. The addition of BMC in TSYE broth also substantially inhibited the growth of *Ps. fluorescens*. However, the MIC (2%, v/v) was higher than that against *Aer. hydrophila* (Fig. 1b). The MIC of BSL against *Ps. fluorescens* was $> 2\%$ (v/v).

The effect of inoculum level (10^3 and 10^6 cfu ml^{-1}) on the MIC of BMC against *Aer. hydrophila* (AFISC 0110) and *Ps. fluorescens* (AFISC 3105) in TSYE broth was also determined at 30 °C using the indirect impedance method (Fig. 1). The MIC of BMC against *Aer. hydrophila* was not affected by the increase of inoculum level from 10^3 to 10^6 cfu ml^{-1} (MIC remained at 0·125%, v/v) (Fig. 1a). However, the MIC of BMC against *Ps. fluorescens* increased to beyond 2% (v/v) (Fig. 1b).

Bactericidal effect of essential oil against resting cells of *Aer. hydrophila* and *Ps. fluorescens* in saline

The effect of BMC (0·1 and 1%, v/v) on resting cells (10^5 cfu ml^{-1}) of *Aer. hydrophila* (AFISC 0110) and *Ps. fluorescens* (AFISC 3105) in saline (0·9% w/v NaCl) was determined after treatment at 20 °C for 10 min (Fig. 2). The addition of either 0·1% or 1% (v/v) BMC reduced the viable count of *Aer. hydrophila* to below the detection limit (< 1 cfu ml^{-1}). The addition of 1% (v/v) BMC was also bactericidal to *Ps. fluorescens* resting cells. However, BMC at 0·1% (v/v) showed no effect on the resting cells of this organism.

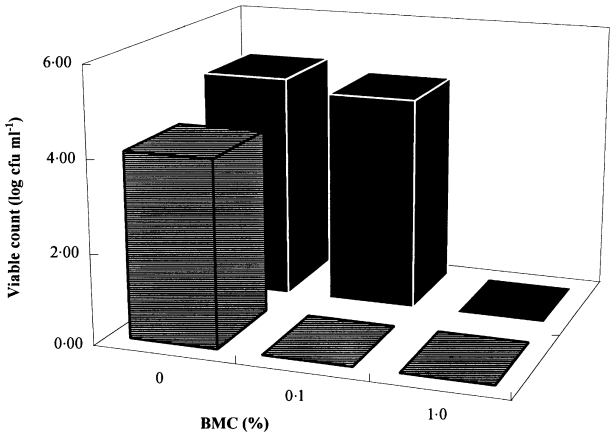

Fig. 2 Residual viable counts of *Aeromonas hydrophila* and *Pseudomonas fluorescens* (each with initial cell concentration of 10^5 cfu ml^{-1}) after treatment with 0, 0·1 and 1·0% (v/v) BMC in saline for 10 min at 20 °C

Effect of BMC on growth of *Aer. hydrophila* and *Ps. fluorescens* in lettuce extract

The inhibitory effect of BMC (0·1 and 1%, v/v) on growth of *Aer. hydrophila* (AFISC 0110) and *Ps. fluorescens* (AFISC 3105) (inoculated at 10^5 cfu ml^{-1}) was also determined in filter-sterilized fresh lettuce supernatant at 10 °C (Fig. 3a, b). The addition of 0·1 and 1% (v/v) BMC to the lettuce supernatant produced a decrease in the viable count of *Aer. hydrophila* from 10^5 to 3 and < 1 cfu ml^{-1}, respectively, within 1 h after the addition of the essential oil (Fig. 3a). At both concentrations, BMC maintained complete inhibition of *Aer. hydrophila* (viable count < 1 cfu ml^{-1}) for the whole duration (14 d) of the experiment (Fig. 3a). The addition of either 0·1 or 1% (v/v) BMC also produced a substantial inhibitory effect on the growth of *Ps. fluorescens*, with a 3 log reduction in viable count at day 14 (Fig. 3b).

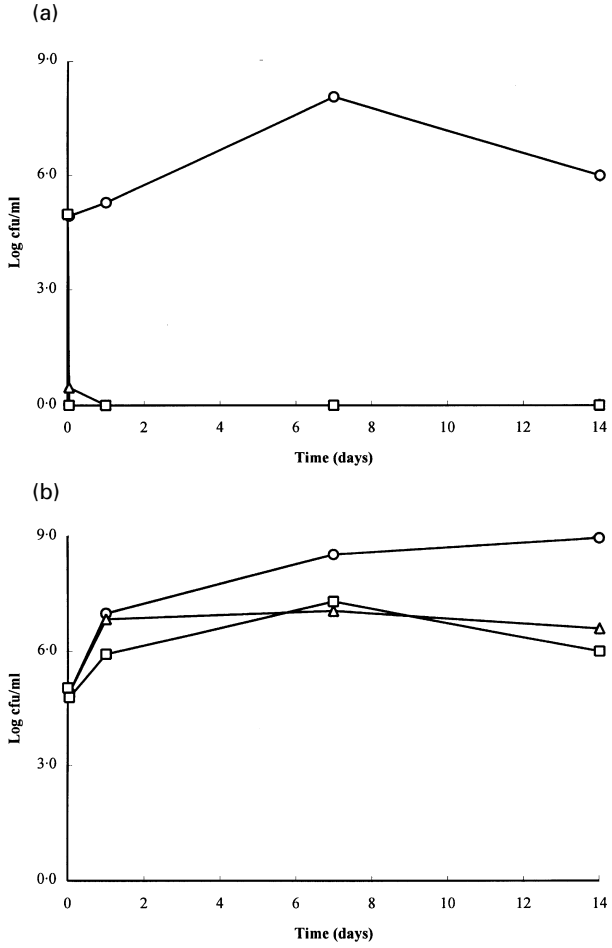

Fig. 3 Viable count of (a) *Aeromonas hydrophila* and (b) *Pseudomonas fluorescens* in filter-sterilized lettuce extract in the presence of 0 (○), 0·1 (△) and 1·0% (□) (v/v) BMC incubated at 10 °C

Microflora of fresh lettuce and survival after washing with suspensions of basil essential oil

Fresh lettuce samples were plated onto PCA, CFC, AA and VRBGA for the enumeration of total viable count and presumptive *Pseudomonas*, *Aeromonas* and Enterobacteriaceae. The effect of washing (20 °C for 10 min) with BMC (0·1 and 1%, v/v) on survival of the natural flora of fresh lettuce leaves was compared with washing with 125 ppm chlorine and sterile distilled water (Table 2). Washing with sterile distilled water had a minimal effect in reducing the viable count on any of the four media used and substantial counts were detected in the collected washings. After washing with 125 ppm chlorine, a 1·5 log reduction in viable count of the lettuce was observed using all four media and no viable cells were detected in the collected washings. Washing lettuce with 0·1% (v/v) BMC produced a 2 log reduction in the viable count on all four media. After washing with 1% (v/v) BMC, less than 200 cfu g^{-1} viable bacteria was detected in lettuce ($>$ 2 log reduction) and less than 20 cfu ml^{-1} viable bacteria was detected in the collected washings.

DISCUSSION

A variety of methodologies has been reported for determination of antimicrobial activity of essential oils depending on the type of essential oils and target organisms. The most frequently used methods included paper disc agar diffusion (Reuveni *et al.* 1984; Gangrade *et al.* 1989; Sinha and Gulati 1990; Meena and Sethi 1994), agar well diffusion (Deans and Ritchie 1987) and incorporation of essential oil in agar media prior to inoculation (Dube *et al.* 1989; Patkar *et al.* 1993; Stecchini *et al.* 1993). In the current study, basil essential oils were also first screened for spectrum of antimicrobial activity using an agar well diffusion method, and then examined in more detail in broth systems using an indirect impedance method and verified in food systems by viable count determinations.

Table 2 Viable count of fresh lettuce leaves after washing with chlorine or BMC

Treatment		Viable count (log cfu g^{-1}) in different media			
		PCA	CFC	AA	VRBGA
Control	Lettuce	5·11	4·54	3·51	4·06
H_2O	Lettuce	4·63	4·1	3·15	4·09
	Washing agent	4·15	3·11	2·09	3·39
Chlorine (125 ppm)	Lettuce	3·76	3·01	$<$2·31	2·31
	Washing agent	$<$1·31	$<$1·31	$<$1·31	$<$1·31
BMC, 0·1%	Lettuce	3·31	2·31	$<$2·31	$<$2·31
	Washing agent	$<$1·31	$<$1·31	$<$1·31	$<$1·31
BMC, 1%	Lettuce	$<$2·31	$<$2·31	$<$2·31	$<$2·31
	Washing agent	$<$1·31	$<$1·31	$<$1·31	$<$1·31

The antibacterial spectrum of basil essential oil obtained in this study is similar to that reported by Deans and Ritchie (1987), showing activity against most of the Gram-positive and Gram-negative bacteria except species of *Clostridium* and *Pseudomonas*. In addition, basil essential oil also shows activity against all the eight strains of yeasts and three strains of moulds tested.

The antimicrobial activity of basil essential oil against *Aer. hydrophila* was examined further in broth, saline and fresh lettuce extract test systems. The MIC of BMC against *Aer. hydrophila* (0·125%) in TSYE broth is in the same range as those of clove oil (0·05%) and coriander oil (0·125%), but lower than those of nutmeg oil (1%) and pepper oil (1·5%) (Stecchini *et al.* 1993). However, this direct comparison may not be appropriate since Stecchini *et al.* (1993) obtained the MICs using an agar test system. Results from the current study also showed that the antimicrobial effect of basil oil against *Aer. hydrophila* is bactericidal and the effect was immediate (within 10 min). This bactericidal effect was also verified in a fresh lettuce extract challenged with *Aer. hydrophila*. These results suggest that basil essential oil could be a beneficial component of salad dressings or product treatments that provide enhanced preservation against *Aer. hydrophila* in MPF lettuce or other vegetable products.

The antimicrobial activity of basil essential oil against *Ps. fluorescens* varied somewhat depending on the testing systems employed. In the agar well diffusion system the growth of *Ps. fluorescens* was unaffected by basil oils. In contrast, in the broth and lettuce extract systems, inhibition of growth occurred in the presence of BMC and in the resting cells system, no viable cells were detected on incubation with 1% (v/v) BMC. However, the antimicrobial effect of BMC against *Aer. hydrophila* was substantially greater than that against *Ps. fluorescens*.

One of the most important steps in the production of MPF salad products is washing the produce with up to 200–300 ppm chlorine at a low temperature (Beuchat 1996) to reduce the microbial load on the product. However, chlorine washing systems can produce harmful by-products (chloramines and trihalomethanes) and there is great interest in developing alternative washing sanitizers. The effectiveness of washing fresh lettuce with 0·1 and 1% (v/v) BMC on total viable count and presumptive counts of *Pseudomonas*, *Aeromonas* and Enterobacteriaceae on lettuce was comparable with that of washing in 125 ppm chlorine. This result indicates that basil or other plant essential oils with antimicrobial activity could play a role in offering a natural alternative for the washing of selected fresh salad produce to replace or

reduce the concentration of chlorine. An alternative approach would be to deliver essential oils to the product as a post-wash application, possibly incorporated into an edible coating (Avena-Bustillos *et al.* 1993), for enhancement of preservation during storage. Such uses of essential oils would be dependant on cost considerations and the original odour and flavour of the oils being appropriate to the final product types.

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