Preservation of Acidified Cucumbers with a Natural Preservative Combination of Fumaric Acid and Allyl Isothiocyanate that Target Lactic Acid Bacteria and Yeasts

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ABSTRACT: Without the addition of preservative compounds cucumbers acidified with 150 mM acetic acid with pH adjusted to 3.5 typically undergo fermentation by lactic acid bacteria. Fumaric acid (20 mM) inhibited growth of *Lactobacillus plantarum* and the lactic acid bacteria present on fresh cucumbers, but spoilage then occurred due to growth of fermentative yeasts, which produced ethanol in the cucumbers. Allyl isothiocyanate (2 mM) prevented growth of *Zygosaccharomyces globiformis*, which has been responsible for commercial pickle spoilage, as well as the yeasts that were present on fresh cucumbers. However, allyl isothiocyanate did not prevent growth of *Lactobacillus plantarum*. When these compounds were added in combination to acidified cucumbers, the cucumbers were successfully preserved as indicated by the fact that neither yeasts or lactic acid bacteria increased in numbers nor were lactic acid or ethanol produced by microorganisms when cucumbers were stored at 30 °C for at least 2 mo. This combination of 2 naturally occurring preservative compounds may serve as an alternative approach to the use of sodium benzoate or sodium metabisulfite for preservation of acidified vegetables without a thermal process.

Keywords: allyl isothiocyanate, benzoic acid, *Cucumis sativus*, fumaric acid, *Lactobacillus plantarum*, sulfite, *Zygosaccharomyces*

Introduction

Vegetables such as peppers (Gabaldón-Leyva and others 2007) and cauliflower (Dakin and Scholey 1970) have for many years been preserved in bulk tanks with acid, high salt (up to 15%), and sulfite as a preservative. More recently, large quantities of smaller sizes of cucumbers are being preserved in barrels to be shipped from growing areas in Asia to processors in Europe and North America in brine solution containing 4% NaCl and 0.5% calcium chloride to preserve firmness along with 2.5% to 3.5% acetic acid and 150 ppm sulfite to prevent microbial growth. Large amounts of high salt and acid waste brines are generated when these preserved vegetables are desalted or deacidified so they are suitable for use in pickled vegetable products. McFeeters (1998), Papageorge and others (2003), and Pérez-Díaz and McFeeters (2008) have shown that peppers and cucumbers can be preserved without salt and with reduced levels of acetic acid by using calcium chloride to preserve texture and sufficient amounts of the traditional preservatives sodium metabisulfite and sodium benzoate to prevent growth of spoilage microorganisms.

Questions have been raised about the use of these preservatives in foods. Sulfites have been shown to cause serious hypersensitivity responses in some people who suffer from asthma (Bush and others 1986). Sodium benzoate when heated in low pH products, such as soft drinks, with ascorbic acid has been found to break down to a small extent to produce benzene in low part per billion concentrations (Gardner and Lawrence 1993; Nyman and others 2008; Poucke and others 2008). As a result some consumers avoid products that contain these compounds and a website (Anonymous 2007) that provides listings of ingredient labels for commercial food products cautions about use of these preservatives. Processors would have the option of avoiding their use in bulk stored vegetables if alternative ingredients could be identified that would prevent spoilage organisms, such as lactic acid bacteria and yeasts from growing in acidified vegetables. Recently, it has been found in our laboratory that fumaric acid in combination with acetic acid in cucumber juice inhibits growth of acid tolerant lactic acid bacteria, but not yeasts. Fumaric acid is an intermediate of the citric acid cycle that is central to the energy metabolism of most aerobic plants, animals, and microorganisms and it is a food ingredient generally recognized as safe (GRAS) by Food and Drug Administration (FDA). However, successful use of fumaric acid for bulk preservation of vegetables without a thermal process would depend upon combination with an inhibitor of yeast growth.

A variety of isothiocyanate compounds are found in *Brassica* vegetables. Some of these isothiocyanates may be beneficial to human health (Traka and Mithen 2009) and have been reported to exert antimicrobial activities. In particular, allyl isothiocyanate (AITC), which forms from the degradation of sinigrin in *Brassica* plants, has been found to have antifungal activity (Mayton and others 1996; Olivier and others 1999) at low concentrations, while being much less inhibitory toward lactic acid bacteria (Shofran and others 1998). AITC is the major component in mustard oil,

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Paper nr FSR09-17 of the Journal Series of the Dept. of Food Science, North Carolina State Univ., Raleigh, N.C. 27695-7624, U.S.A. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Dept. of Agriculture or North Carolina Agricultural Research Service, or approval to the exclusion of other products that may be suitable.

doi: 10.1111/j.1750-3841.2010.01587.x

THE Society for Food Science & Technology
Journal of Food Science
A Publication of the Institute of Food Technologists

which is used as a flavoring in foods. Therefore, AITC was evaluated for its ability to inhibit potential spoilage yeasts in acidified cucumbers in combination with fumaric acid to inhibit lactic acid bacteria.

The objective of this study was to determine if acidified cucumbers stored in conditions similar to those in which sodium benzoate is an effective preservative (Pérez-Díaz and McFeeters 2008) could be made microbiologically stable by the use of a combination of fumaric acid and AITC.

Materials and Methods

Size 2B cucumbers (25.4 to 31.8 mm dia) were obtained from a local processor and packed without washing into 46 oz jars. For all experiments, triplicate jars of cucumbers were prepared for each treatment. The cucumber : brine ratio in the jars was 55 : 45 (w/v). Cover solutions contained either 190 or 333 mM acetic acid from commercial vinegar (20% acetic acid) and 222 mM calcium chloride to maintain the firmness of cucumbers during storage. Equilibrated jars of cover brine solution and cucumbers contained either 85 or 150 mM acetic acid and 100 mM calcium chloride. Weighed amounts of dry sodium benzoate (Fisher Scientific Co., Pittsburgh, Pa., U.S.A.), potassium sorbate (Fisher Scientific Co.), and sodium fumarate (Alfa Aesar, Ward Hill, Mass., U.S.A.) were added to the 46 oz jars of acidified cucumbers to give the intended equilibrated concentrations of these preservative. Liquid allyl isothiocyanate (Sigma-Aldrich Co., St. Louis, Mo., U.S.A.) was directly added into the jars.

Any required pH adjustments so the initial equilibrated pH of the brined cucumbers was 3.5 ± 0.1, were done by addition of less than 10 mL of 3 N HCl or 5 N NaOH solution. The amount of HCl or NaOH required was determined by mixing cucumber slurry and cover brine solution 55 : 45 (w/v) along with the required preservative. Then the mixture was titrated with acid or base to the target pH. Jars were closed with commercial metal lug caps fitted with a rubber septum to allow for inoculation of microorganisms and sampling of the jars with sterile syringes. The lids were heated in boiling water for 15 s to soften the sealing compound and immediately applied to the filled jars. Jars were incubated at 30 °C. Initial samples of brine for chemical and microbiological analyses were taken from the jars after a 4-d equilibration period. Samples were then collected during the storage period to evaluate whether microbial growth occurred.

A treatment was judged to be effective for preserving cucumbers only if there was no detectable microbial growth in all jars with the treatment. Jars were visually monitored for the development of turbidity in the cover solution and for pressure on the lids. After turbidity developed in a container, brine samples were taken using aseptic techniques for microbiological analysis. Samples were plated on deMan Rogosa and Sharpe agar (MRSA) or Yeast and Mold agar supplemented with 1 mM chlortetracycline and 1.5 mM chloramphenicol (YM/A) for bacteria and yeasts, respectively. Plates were incubated at 30 ˚C for 72 h. MRS plates were incubated under anaerobic conditions. Additionally, occurrence of microbial growth was also evaluated by determination of changes in sugars, organic acids, and ethanol during incubation of samples at 30 °C. Analyses were done by high-performance liquid chromatography (HPLC) on a 30 cm HPX-87H column (Bio-Rad Laboratories, Hercules, Calif., U.S.A.) (McFeeters and Barish 2003). The column was heated to 65 °C and eluted with 0.03 N sulfuric acid at a flow rate of 1.0 mL/min. A Thermo Separations UV6000 diode array detector set to collect data at 210 nm was used to detect organic acids. A Waters model 410 refractive index detector connected in series was used to measure glucose, fructose, and ethanol.

Preservation treatments in which there was no detectable microbial growth after 3 wk of incubation were challenged by inoculating *Lactobacillus plantarum* LA0445, and a cocktail of 5 *Zygosaccharomyces globiformis* (SPYs) isolates. *L. plantarum* LA0445 was originally isolated from fermented cucumbers with a pH of 3.3 (Fleming and others 1988). The 5 *Z. globiformis* strains used were isolated from spoiled sweet pickles (Bell and Etchells 1952). Bacterial and yeast cultures were propagated in MRSA or YM broth, respectively. The microorganisms were inoculated through the lid septum to give initial populations of 10^5 CFU/g *L. plantarum* and 10^4 CFU/g of the 5 strains of *Z. globiformis* in each jar. Control jars were not inoculated.

Removal of the natural vegetative microorganisms from the fresh cucumbers for some experimental treatments was accomplished by pasteurizing the sealed jars in a water bath in a steam jacketed kettle to a temperature of 74 °C at the slowest heating point in the jars for 15 min. Subsequently, jars were cooled to 40 °C in cold tap water before removing them from the steam jacketed kettle.

Results and Discussion

Fresh cucumbers were stored in jars with 85 mM acetic acid, 30 mM calcium chloride, pH adjusted to 3.5 and variable amounts of fumaric acid to determine the effect of the fumaric acid concentration on the ability of the naturally occurring lactic acid bacteria and yeasts to grow and produce lactic acid and ethanol from sugars present in the fresh cucumbers. From an initial population of 10^4 CFU/g LAB and 10^2 CFU/g yeasts Figure 1 shows that after 5 d at 30 °C the lactic acid bacteria increased to 10^9 CFU/g and yeasts to 10^5 CFU/g in the absence of added fumaric acid. There was less growth of lactic acid bacteria (10^6 to 10^7 CFU/g) when 10 and 40 mM fumaric acid was added, but the yeast population still increased to 10^5 CFU/g. However, with 60 mM fumaric acid both groups of microorganisms were reduced in numbers compared to the fresh cucumbers, similar to a control treatment with 12 mM sodium benzoate added as preservative. Table 1 shows the formation of ethanol and lactic acid in these acidified cucumbers 4 wk postpacking. Ethanol and lactic acid were produced in the stored cucumbers with 10 and 40 mM fumaric acid as well as

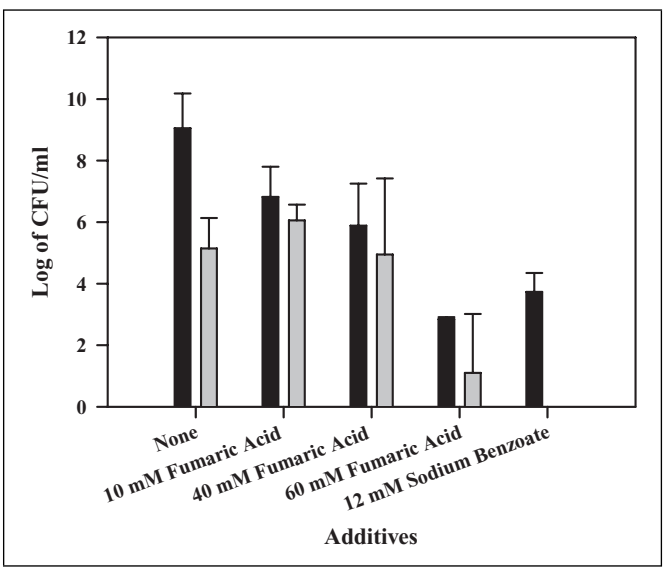

Figure 1—Effect of fumaric acid on growth of lactic acid bacteria and yeasts in brined cucumbers. Cucumbers were brined with 85 mM acetic acid and fumaric acid or sodium benzoate. Numbers of lactic acid bacteria (■) and yeasts (■) were determined 5 d postpacking.

the cucumbers without added preservative. This showed that these concentrations of fumaric acid did not stop fermentative activity by either the naturally present lactic acid bacteria or yeasts with this low concentration of acetic acid as the primary acidulant. At its solubility limit of 60 mM, however, fumaric acid prevented production of both lactic acid and ethanol just as occurred when 12 mM sodium benzoate was used as a preservative (Pérez-Díaz and McFeeters 2008).

The ability of fumaric acid to prevent growth of inoculated *L. plantarum* and *Z. globiformis* was investigated by pasteurizing jars of cucumbers after brining them to contain 150 mM acetic acid, 20 mM calcium chloride with and without 20 mM fumaric acid at pH 3.5. The pasteurization treatment killed the naturally occurring vegetative microorganisms on the cucumbers so only the inoculated organisms were present. Analysis of brine samples after 4 wk storage at 30 °C showed that 20 mM fumaric acid prevented lactic acid production even with a large inoculum of the very acid resistant *L. plantarum,* which is capable of carrying out a complete fermentation of cucumbers that are brined with 6% NaCl (McDonald and others 1993). In 150 mM acetic acid, but without added fumaric acid 65 mM lactic acid was produced by the inoculated *L. plantarum* (Table 2). However, 20 mM fumaric acid with 150 mM acetic acid did not prevent ethanol production by the inoculated yeast strains. This finding confirms the previous observation on the ability of fumaric acid to prevent growth of lactic acid bacteria; but not yeasts in a cucumber juice medium containing acetic acid and calcium chloride (Pérez-Díaz 2009).

Given the ability of fumaric acid to inhibit growth of lactic acid bacteria in combination with acetic acid, the possibility of preserving cucumbers with the organic acids and a yeasts inhibitory agent was tested. It was previously observed that 12 mM sodium benzoate preserved cucumbers, but if only 3 or 6 mM benzoate were added, a spoilage characterized by formation of lactic acid, but not ethanol proceeded (Pérez-Díaz and McFeeters 2008); which suggested that at these lower concentrations sodium benzoate inhibited yeast growth. Therefore, fumaric acid was added in combination with 3 mM and 6 mM sodium benzoate. The results, however, showed that ethanol, indicative of yeast fermentation, was produced with both concentrations of sodium benzoate added in combination with either 30 or 40 mM fumaric acid, even though 12 mM sodium benzoate without added fumaric acid prevented production of both lactic acid and ethanol (Table 3).

Shofran and others (1998) observed that AITC had much lower minimum inhibitory concentrations against several yeast species compared to a number of bacterial species in tryptic soy broth (pH approximately 7), although it did not inhibit 3 species of *Zygosaccharomyces* yeasts, including *Z. globiformis* at neutral pH. Based upon these observations AITC was evaluated for its ability to preserve acidified cucumbers in combination with fumaric acid. Table 4 shows that after 8 wk incubation pasteurized acidified cucumbers inoculated with both *L. plantarum* and *Z. globiformis* contained 60 mM ethanol when 20 mM fumaric acid was added without AITC, 74 mM lactic acid when 2.0 mM AITC was added without fumaric acid and nondetectable levels of both ethanol and lactic acid when both fumaric acid and AITC were added to jars. These results showed that fumaric acid inhibited growth of *L. plantarum*, AITC inhibited growth of *Z. globiformis*, and that when both compounds were added the cucumbers were microbiologically stable.

Table 5 shows results when fumaric acid and AITC were added alone or in combination to nonpasteurized acidified cucumbers. Lactic acid was produced with only a small amount of ethanol when neither compound was added. This is similar to cucumber fermentations that rely upon growth of the naturally occurring lactic acid bacteria where the naturally occurring yeasts on the cucumbers typically show little or no growth. Surprisingly, no lactic acid production and minimal ethanol production occurred in the nonpasteurized acidified cucumbers when either 20 mM fumaric acid

Table 1 — Organic acids and sugars profile of cucumbers brined with 85 mM acetic acid, 100 mM calcium chloride, and fumaric acid (pH 3.5 ± 0.1).

	Substrates utilized (mM)			Products formed (mM)	
Additives	Fumaric acid	Glucose	Fructose	Ethanol	Lactic acid
			A. Two wk postpacking		
None	—	9.2 ± 2.1	11.1 ± 4.7	11.10 ± 3.3	44.5 ± 5.4
12 mM benzoic acid	—	None	None	5.93 ± 0.2	ND
10 mM fumaric acid	None	None	None	15.6 ± 10.2	ND
40 mM fumaric acid	None	None	None	7.11 ± 0.5	ND
60 mM fumaric acid	None	None	None	6.96 ± 0.9	ND
			B. Four wk postpacking		
None	—	29.5 ± 5.6	37.6 ± 5.4	98.5 ± 10.3	81.3 ± 4.3
12 mM benzoic acid	—	None	None	3.5 ± 0.3	ND
10 mM fumaric acid	3.8 ± 0.4	28.4 ± 0.6	35.9 ± 2.5	92.8 ± 2.1	39.1 ± 12.9
40 mM fumaric acid	37.7 ± 3.1	30.5 ± 2.2	35.4 ± 1.9	34.3 ± 4.5	72.7 ± 24.5
60 mM fumaric acid	None	None	None	6.3 ± 3.7	ND

ND = not detected.

Table 2 — Growth of *Lactobacillus plantarum* and cucumber spoilage yeasts in pasteurized jars brined with 150 mM acetic acid, 100 mM calcium chloride, and 0 or 20 mM fumaric acid at pH 3.5.

		Substrates utilized (mM)		Products formed (mM)	
Inoculates	Fumaric acid added (mM)	Glucose	Fructose	Ethanol	Lactic acid
None	0	None	None	3.3 ± 0.2	ND
Lactobacillus plantarum	0	None	14.3 ± 0.9	3.6 ± 0.1	65.2 ± 3.6
Zygosaccharomyces globiformis	0	None	29.3 ± 1.4	53.5 ± 0.2	ND
Lactobacillus plantarum	20	None	None	3.1 ± 0.2	ND
Zygosaccharomyces globiformis	20	None	30.5 ± 1.3	52.8 ± 0.9	ND

ND = not detected.

Table 3—Cucumber preservation by 150 mM acetic acid, 30 or 40 mM fumaric acid, 100 mM $CaCl_2$, and low amounts of sodium benzoate at pH 3.5.

Treatment identification	Fumaric acid added (mM)	Sodium benzoate added (mM)	Substrates utilized (mM)		Products formed (mM)	
			Glucose	Fructose	Ethanol	Lactic acid
1	None	None	17.5 ± 1.6	21.2 ± 2.2	21.6 ± 7.9	72.3 ± 28.8
2	0	12	None	None	ND	ND
3	30	3	None	37.6 ± 2.6	78.7 ± 0.2	ND
4	40	3	2.2 ± 1.2	33.4 ± 7.6	70.9 ± 5.1	ND
5	30	6	None	18.8 ± 7.3	53.1 ± 8.7	ND
6	40	6	None	22.2 ± 3.8	68.4 ± 5.9	ND

ND = not detected.

Table 4—Ability of allyl isothiocyanate and fumaric acid to inhibit microbial[a] production of ethanol and lactic acid in pasteurized cucumber jars at pH 3.5.

Fumaric acid added (mM)	Allyl isothiocyanate (mM)	Substrates utilized (mM)		Products formed (mM)	
		Glucose	Fructose	Lactic acid	Ethanol
0 (12 mM benzoate)	0	None	None	ND	ND
20	0	None	27.4 ± 0.4	ND	60.7 ± 3.0
0	2	28.2 ± 15.1	10.3 ± 9.6	74.2 ± 25.2	3.8 ± 3.3
20	2	None	None	ND	ND

[a] *Lactobacillus plantarum* and the *Zygosaccharomyces globiformis* cocktail were inoculated in the cucumber jars to 104 and 103 CFU/g, respectively.
ND = not detected.

Table 5—Ability of allyl isothiocyanate and fumaric acid to inhibit microbial productions of ethanol and lactic acid in nonpasteurized cucumber jars at pH 3.5.

Fumaric acid added (mM)	Allyl isothiocyanate added (mM)	Inocula *L. plantarum*/ *Zygosaccharomyces* spp. (CFU/mL)	Substrates utilized (mM)		Products formed (mM)	
			Glucose	Fructose	Lactic acid	Ethanol
0	0	None	16.5 ± 6.5	23.4 ± 7.0	40.1 ± 10.1	10.8 ± 2.5
0 (12 mM benzoate)	0	None	None	None	ND	3.0 ± 1.3
20	0	None	None	None	ND	11.5 ± 1.1
0	2	None	None	10.4 ± 1.7	ND	9.0 ± 0.8
5	2	None	None	None	ND	14.2 ± 9.9
20	0.5	None	None	None	ND	12.3 ± 1.7
5	2	$10^4/10^3$	None	None	ND	ND
20	2	$10^5/10^4$	None	None	ND	ND
20	0.5	$10^4/10^3$	None	18.3 ± 2.4	ND	36.8 ± 2.4
20	1	$10^4/10^3$	None	11.1 ± 3.6	ND	19.7 ± 5.4
20	3	$10^5/10^4$	None	None	ND	ND

ND = not detected.

or 2.0 mM AITC was used alone. This suggests that fumaric acid has some inhibitory activity against the naturally occurring microflora on the cucumbers and against yeasts in addition to lactic acid bacteria. Similarly, AITC may have some inhibitory activity against some naturally occurring lactic acid bacteria on the fresh cucumbers in addition to preventing growth of yeasts. If both fumaric acid and AITC were added to the jars of cucumbers, the cucumbers were preserved. Finally, when *L. plantarum* and *Z. globiformis* were added to nonpasteurized acidified pickles there was no production of either lactic acid or ethanol with fumaric acid added at either 5 or 20 mM in combination with AITC added at 2.0 mM or higher. Ethanol was produced when 0.5 or 1 mM AITC was added to cucumbers in combination with 20 mM fumaric acid. This showed that 20 mM fumaric acid prevented growth of any lactic acid bacteria, but that 1.0 mM AITC was not a sufficient amount to prevent growth of the *Z. globiformis* yeast strains.

Prevention of growth of *Zygosaccharomyces* spp. in foods, including acid and acidified products, has historically represented a challenge for manufacturers due to the capacity of these yeasts to tolerate high levels of preservatives such as sorbic acid (up to 9 mM), benzoic acid (approximately 3 mM), acetic acid (up to 555 mM), and ethanol (1950 mM) at pH 4.0 (Martorell and others 2007). In these experiments, 6 mM sodium benzoate at pH 3.5 in combination with 150 mM acetic acid did not prevent growth of *Z. globiformis*. Additionally, members of this genus are resistant to heat, particularly in products with high sugar content. Both *Z. globiformis* (Bell and Etchells 1952) and *Zygosaccharomyces rouxii* (unpublished) have been isolated from commercial sweet cucumber products. The results presented here showed that 2.0 mM AITC in combination with acetic acid at pH 3.5 is effective in preventing growth of the *Z. globiformis* strains isolated from sweet pickles.

Conclusions

Cucumber preservation may be achieved without a thermal treatment by storing cucumbers in a brine solution that contains 150 mM acetic acid, at pH 3.5 in combination with 20 mM fumaric acid to prevent growth of LAB and 2.0 mM AITC to prevent growth of yeasts, including very acid and preservative resistant *Zygosaccharomyces* species. These results provide a basis for preservation of acidified vegetables without a thermal process and without use of sodium benzoate or sodium metabisulfite as preservatives.

Acknowledgments

The authors thank Mr. D. Seth Fornea and Ms. Janet Hayes at the USDA-ARS, SAA Food Science Research Unit located in Raleigh, N.C., U.S.A. for excellent technical assistance with HPLC analysis and Mount Olive Pickle Co. for its contribution to this study.

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