Food Processing & Technology

Lingham et al., J Food Process Technol 2012, S11
http://dx.doi.org/10.4172/2157-7110.S11-001

Research Article Open Access

Antimicrobial Activity of Vinegar on Bacterial Species Isolated from Retail and Local Channel Catfish (*Ictalurus punctatus*)

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Abstract

The use of vinegar was studied to reduce the growing number of illnesses caused by spoilage bacteria. The antimicrobial activity of organic vinegar was studied on various bacterial species isolated from domestic channel catfish fillets (*Ictalurus punctatus*). The effectiveness of the vinegar on the bacteria was measured. Bacteria isolated from catfish fillets with the largest inhibition zone were identified through 16S rDNA sequencing to better understand the spoilage bacteria that could be inhibited by vinegar to increase the quality of fishery products. Microbial changes of catfish fillets were evaluated following treatments of vinegar marinades during storage at 4°C and sensory evaluation was performed with baked catfish fillets to evaluate consumer sensory acceptability.

Fish and chip vinegar was the most effective on *Shewanella putrefaciens* isolated from catfish fillets from the market and the pond. Microbial changes were evaluated and sensory evaluation was performed on different acetic acid dilutions of vinegar. Results showed that vinegar diluted to 0.5% acetic acid on catfish fillets would be suitable for prolonging shelf life and appealing to consumers. Vinegar as a natural antimicrobial product can improve the shelf life and safety of food products providing acceptable sensory quality at an affordable price and reducing economic lost due to spoiled catfish and other food products.

Keywords: Antimicrobial activity; Vinegar; Catfish; Bacterial identification

Introduction

There is an increasing interest in applying natural antimicrobial compounds in the food industry. Consumers are increasingly avoiding the consumption of foods treated with chemicals. Natural alternatives are needed to achieve a high level of safety with respect to foodborne pathogenic microorganisms [1]. The natural sanitizers, such as organic acids, have been investigated because of their bactericidal activity [2]. Among the natural products, vinegar, also known as acetic acid, contains sanitizing properties. Vinegar is an acidic liquid that is made from the fermentation of an alcoholic beverage mainly wine [3]. The total acidity of vinegar is expressed as acetic acid which is the major organic acid in vinegar. Acetic acid is a monocarboxylic acid. It has a pungent odor and flavor. It is generally regarded as safe (GRAS) for general purpose and miscellaneous usage. According to Malicki, organic acids are considered weak acids meaning the antimicrobial effect of organic acids is mainly caused by its undissociated forms [4]. They passively diffuse through the bacteria cell wall and internalizing into neutral pH dissociating into anions and protons. Release of the protons causes the internal pH to decrease which exert inhibitory effects on the bacteria [5].

Organic acids have been approved by the Food Safety and Inspection Service of the United States Department of Agriculture [6]. Various researchers have proved the antibacterial effect of organic acids on different types of pathogenic bacteria. Organic acids such as tartaric, citric, lactic, malic, propionic, and acetic acids have been used for years for decontamination of bacteria on beef, pork, and poultry [7]. Organic acids that are used to inhibit spoilage bacteria in meat are applied by spraying and dipping techniques [8]. In a study conducted by Bradley, the addition of citric acid and acetic acid each reduced the growth of Enterobacteriaceae [9].

Chaff vinegar has been found to inhibit the growth of pathogenic bacteria such as *E. coli* [10]. Vinegar assists in suppressing the anthracnose rot in tomatoes [11]. It also assists in eliminating *Salmonella* Typhimurium in carrots [12]. Vinegar may be used as a mixture or alone as a natural flavoring in some salads [13]. These salad dressings provide a harsh environment for foodborne pathogens such as *Salmonella* and *E. coli* to survive because of the acetic or citric acids [14]. In a study by Frederick et al. results indicated that 2% acetic acid reduced the incidence of *Salmonella* on pork [15]. In the United States, salad dressings maintain a good safety record and are widely used [16].

Channel catfish contains health benefits and a wide consumer acceptance. Consuming fish at least one to two times per week results in a positive effect on one's health [17-19]. If not preserved in a certain way, significant economic losses could result from the highly perishable nature of catfish [20]. It is estimated that one-fourth of the world's food supply is lost through microbial activity alone [21]. Freshness of fish deteriorates rapidly so maintaining the quality is of high importance.

Food spoilage can be considered as "any change which renders a product unacceptable for human consumption" [22]. Trimethylamine (TMA) is responsible for the "fishy" odor [23]. When oxygen levels are depleted, trimethylamine oxide (TMAO) is reduced to TMA [24]. Food spoilage is an economical problem despite the food technology and preservation techniques available. CO_2 has a positive effect on

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Received June 01, 2012; **Accepted** September 21, 2012; **Published** September 28, 2012

Citation: Lingham T, Besong S, Ozbay G, Lee JL (2012) Antimicrobial Activity of Vinegar on Bacterial Species Isolated from Retail and Local Channel Catfish (*Ictalurus punctatus*). J Food Process Technol S11-001. doi:10.4172/2157-7110.S11-001

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shelf life. CO_2 and MAP (modified atmosphere packaging) reduces the growth of *Shewanella* and *Pseudomonas* [25-28]. Using CO_2 alone would cause it to dissolve in meat and fish resulting in package shrinking and deformation [29-30]. A disadvantage of MAP is that the increased package volume would increase transportation costs [25]. With vacuum packaging, there is less volume but it is less effective than MAP [26].

To the best of our knowledge, studies have been mostly interested in the inhibitory effect of vinegar on foodborne pathogens and not bacteria isolated from catfish. The objectives of this study were: (1) to measure the antimicrobial activity of vinegar on bacteria isolated from catfish from a local seafood market and local aquaculture pond by performing zone of inhibition test and also evaluating microbial changes following the treatment of vinegar on catfish fillets. (2) To identify the bacteria derived from catfish fillets using 16S rDNA and (3) To evaluate consumer sensory acceptability with catfish fillets with vinegar marinades.

Materials and Methods

Fish and chip (fc) vinegar

Crosse and Blackwell Old English Fish and Chip 100% Natural Malt (FC) Vinegar was used in this study. It was purchased from a retail store. It contains 5% acetic acid by volume. This vinegar product was chosen because it contains 100% natural malt vinegar. Dilutions of fish and chip vinegar to 0.5, 1, 2.5, and 5% acetic acid were used.

Isolation of bacteria from catfish fillets (*Ictalurus punctatus*)

Domestic catfish fillets (*Ictalurus punctatus*) were used. Catfish fillets came from a local seafood market and the Delaware State University Aquaculture Research and Demonstration Facility. Catfish fillets were preserved in ice prior to experiment and were used for positive control. Bacteria derived from catfish fillets were isolated by homogenizing a fillet in 0.85% saline solution in a stomacher bag and spread plating onto Tryptic Soy Agar (TSA) plates. Plates were incubated overnight at 28°C. This was done for two cycles every 2 days until the spoilage point was reached. Bacteria with different morphologies were selected and used in this study to test the inhibitory effect of vinegar on different bacteria isolated from catfish fillets.

Zone of inhibition

The zone of inhibition test measured the antimicrobial activity of FC vinegar on 240 bacteria isolates from catfish fillets. The vinegar sample was filtered prior to testing with 0.22 µm filters (Millex GS Filter Unit, Corrigwohill Co., Cork, Ireland). Each bacterium was inoculated in Tryptic Soy Broth (TSB) for 14 to 16 hours prior to the test to ensure that fresh bacteria were to be tested. Saline solution of 0.85% was used to fix the optical density to 0.3 CFU/ml at 600 nm. One hundred µl of each bacterium was spread onto nutrient agar plates. Two layers of 6 mm discs were placed onto the nutrient agar plates. Discs consisted of distilled water as a negative control and treatments with the dilutions of fish and chip vinegar to 0.5, 1, 2.5, and 5% acetic acid. Twenty µl of the vinegar was placed onto the discs. Each plate was incubated at 28°C. The zones of inhibition were measured in diameter with an Ultra-Tech Carbon Fiber Composite Digital caliper (Penn Tool Co., Maple, NJ). Zone of inhibition was performed in triplicates.

16S rDNA Sequencing

The ten bacteria isolated from catfish fillets from both the retail store and local aquaculture pond that showed the largest inhibition zone sensitive to 5% acetic acid of FC vinegar were selected to be identified in this study. The twenty candidates were identified through 16S rDNA sequencing. Each candidate was streaked on TSA plates and incubated overnight at 28°C to grow pure colonies. Colonies were delivered for 16S rDNA sequencing performed by GENEWIZ Inc. (South Plainfield, NJ). Seven hundred to one thousand base pairs of each 16S rDNA sequencing data were used to search for bacterial species using the Genbank database (www.ncbi.nlm.nih.gov/genbank). Bacterial identity was chosen based on 99% or greater matches.

Bacterial Enumeration

Domestic catfish fillets were purchased from a retail store in Dover, Delaware. Catfish fillets were preserved in ice prior to experiment and were used for positive control. Fillets were cut and 100 g were added to five stomacher bags as follows: control, FC 0.03, FC 0.05, FC 0.5, and FC 5%. Acetic acid dilutions of FC vinegar (250 ml) were poured into each of the five stomacher bags. The stomacher bags were stored at 4°C for 15 minutes to marinate the fillets in the vinegar. Excess vinegar was drained out and 10 g of fillet and 90 ml of 0.85% saline solution were homogenized into each of the five stomacher bags. Ten-fold serial dilutions were performed using TSB and spread onto TSA plates and incubated for 2 days at 25°C. Total bacteria were enumerated. The nine stomacher bags were plated every 2 days until samples reached their spoilage point and the stationary phase of bacterial growth was reached. Bacterial enumeration was performed in triplicates.

Sensory evaluation

Frozen catfish fillets were purchased from a retail store in Dover, Delaware. Fillets were placed in zip lock bags in the following order: control, FC 0.05, FC 0.5, and FC 5%. The control in this study was the same fillet used for the vinegar treated samples, but did not contain any vinegar. Vinegar with different acetic acid dilutions were poured into each of the four zip lock bags. The bags were stored at 4°C for 15 minutes to marinate in the vinegar. Excess vinegar was drained out and the fillets were placed on baking pans. Each sample was baked in separate ovens to avoid cross-contamination of aromas and flavors between samples. They were baked at 176.7°C for 10 minutes per inch of thickness or until flaky and with an opaque appearance. Fillets were cut into small cubes (3 cm^3) using a sterile kitchen knife and placed into small sampling cups. The four samples were labeled with 3-digit random numbers. Twenty-one panelists participated in the sensory evaluation. Each panel received the five samples and distilled water

Market Catfish Isolate			Inhibition zone at various dilutions (mm)			
Cycle	Bag	Bacteria	0.5%	1%	2.5%	5%*
1	1	2	4.41 ± 0.08	5.14 ± 0.08	7.83 ± 0.03	12.30 ± 0.03
2	1	8	2.27 ± 0.02	3.86 ± 0.08	10.37 ± 0.03	12.17 ± 0.01
1	3	4	3.34 ± 0.07	5.26 ± 0.05	9.39 ± 0.08	11.63 ± 0.04
1	2	4	2.44 ± 0.09	6.95 ± 0.06	9.87 ± 0.04	11.55 ± 0.03

Pond Catfish Isolate			Inhibition zone at various dilutions (mm)			
Cycle	Bag	Bacteria	0.5%	1%	2.5%	5%*
1	5	2	3.58 ± 0.08	6.29 ± 0.07	10.64 ± 0.05	14.98 ± 0.06
2	2	1	5.70 ± 0.04	7.58 ± 0.03	6.57 ± 0.04	11.98 ± 0.02
2	3	1	2.82 ± 0.03	3.75 ± 0.02	6.89 ± 0.03	10.39 ± 0.04
1	2	6	0.96 ± 0.07	2.36 ± 0.02	6.18 ± 0.01	9.08 ± 0.05

*Ranked from largest to smallest zone of inhibition by 5% acetic acid dilution of fish and chip vinegar.

Table 1: Bacteria isolated from catfish from the retail store and local aquaculture pond with the largest zone of inhibition from fish and chip vinegar.

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to cleanse their pallet after each sample. Appearance (color), flavor, aroma and texture were evaluated using the Hedonic nine point scale; 1= Dislike extremely and 9= Like extremely.

Statistical analysis

Microsoft Excel (1997) was used to analyze the bacterial data. All sensory evaluation data were analyzed using generalized linear models generated by the SAS System ('SASApp', X64_SRV08, SAS Institute, Cary, NC). Rating was the dependent variable. The analysis was based on the Hedonic nine-point scale of attributes including appearance (color), aroma, flavor, and texture per sample (Control, FC 0.05, FC 0.5, and FC 5%). A confidence interval of 95% and alpha level (α) 0.05 was used for the statistical analysis. Values of $p<0.05$ were deemed significant.

Results and Discussion

Determination of antimicrobial activity by using zone of inhibition method

This was the only study that evaluated the inhibitory effect of acetic acid from vinegar on bacteria isolated from catfish. Various acetic acid dilutions of FC vinegar were tested on bacteria isolated from market and local aquaculture pond catfish fillets. The zones of inhibition were ranked according to 5% acetic acid of vinegar. The largest zone of

Catfish Isolate			Bacterial Identification	Similarity (%)[1]	Length (bp)[2]	GenBank Accession Number
Cycle	Bag	Bacteria	16S rDNA Sequencing Result			
2	1	2	*Shewanella putrefaciens*	100	840	AB681550.1
2	1	7	*Shewanella putrefaciens*	100	840	AB681550.1
2	3	4	*Shewanella putrefaciens*	100	770	FJ375179.1
2	2	4	*Shewanella putrefaciens*	99	764	AB681550.1
1	1	2	*Aeromonas sobria*	100	840	JN55613.1
1	2	3	*Aeromonas sobria*	99	839	JN55613.1
1	6	1	*Shewanella putrefaciens*	100	818	NR 044863.1
2	2	1	*Shewanella putrefaciens*	100	840	AB681550.1
1	3	3	*Enterobacteriaceae bacterium/ Rahnella aquatilis*	100 99	840 839	HQ824881.1 DQ440548.1
1	4	1	*Pseudomonas fragi*	100	827	AB685609.1

[1]Similarity of 16S rDNA region between catfish isolate samples and closest relative found in GenBank database
[2]Base pair used for gene alignment

Table 2: Identification of bacterial isolates from local seafood market catfish based on 16S rDNA sequencing.

Catfish Isolate			Bacterial Identification	Similiarity (%)[1]	Length (bp)[2]	GenBank Accession Number
Cycle	Bag	Bacteria	16S rDNA Sequencing Result			
1	5	2	*Shewanella putrefaciens*	99	837	AB208055.1
1	2	1	*Stenotrophomonas maltophila/ Stenotrophomonas rhizophia*	100 100	910 910	HM007572.1 HQ831393.1
1	3	1	*Shewanella putrefaciens*	99	769	AB208055.1
1	2	6	*Aeromonas hydrophila/ Aeromonas veronii*	100 100	840 840	HE681732.1 JQ301790.1
1	2	2	*Pantoea agglomerans*	99	766	FJ357834.1
1	1	3	*Plesiomonas shigelloides*	99	839	HM007572.1
1	5	3	*Pseudomonas fluorescens/ Pseudomonas mandelii*	100 100	840 840	AB681956.1 JN638055.1
1	1	1	*Plesiomonas shigelloides*	100	770	FJ375179.1
1	3	3	*Pantoea agglomerans*	99	697	AJ233423.1

[1]Similarity of 16S rDNA region between catfish isolate samples and closest relative found in GenBank database.
[2]Base pair used for gene alignment.

Table 3: Identification of bacterial isolates from local aquaculture pond catfish based on 16S rDNA sequencing.

Zone of inhibition with FC 5% (mm)	Bacterial identification	Number of bacteria*
10-15	*Shewanella putrefaciens*	8
	Stenotrophomonas maltophila/Stenotrophomonas rhizophia	1
	Aeromonas sobria	2
	Aeromonas hydrophila/ Aeromonas veronii	1
8-10	*Enterobacteriaceae bacterium/Rahnella aquatilis*	1
	Pseudomonas fragi	1
6-8	*Pantoea agglomerans*	2
	Plesiomonas shigelloides	2
	Pseudomonas fluorescens/Pseudomonas mandelii	1
	Pseudomonas putida/Pseudomonas veronii	1

*The frequency of each catfish bacterial isolate identified from the retail store and pond

Table 4: Identification of bacterial isolates from the retail store local aquaculture pond based on 16S rDNA sequencing with zone of inhibition by fish and chip vinegar with 5% acetic acid.

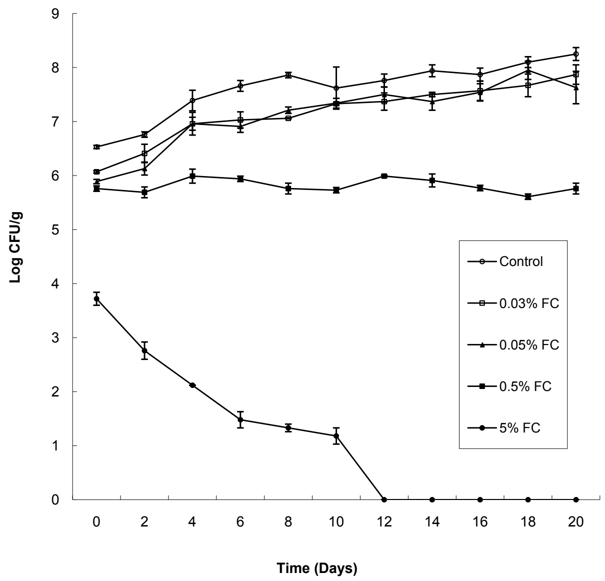

Figure 1: Changes in total bacterial count from catfish fillet with control with no vinegar and vinegar treatments. Control: catfish fillets were preserved in ice prior to experiment. Vinegar treatments included 0.03, 0.05, 0.5 and 5% acetic acid of fish and chip vinegar during storage at 4°C for 20 days.

Treatment	Appearance	Aroma	Flavor	Texture
Control	5.29	5.62	6.05	6.43
FC 0.05%	5.33	5.19	5.57	5.52
FC 0.5%	5.71	5.62	5.29	5.38
FC 5%	5.33	*3.71	*4.00	5.00

*Aroma and flavor of sample FC 5% are significantly different ($p < 0.05$).

Table 5: Average ratings for appearance (color), aroma flavor, and texture of baked catfish fillet with treatment of 0.05, 0.5 and 5% acetic acid of fish and chip vinegar.

inhibition of market catfish from FC vinegar was 12.30 mm in diameter (Table 1) and was higher from the pond measuring at 14.98 mm in diameter (Table 1). FC vinegar exerted antimicrobial activity at all acetic acid dilutions of vinegar (0.5, 1, 2.5 and 5%) tested in the retail and pond catfish.

Bacterial identification

Bacteria with the largest zone of inhibition from FC vinegar, from both the market and local aquaculture pond catfish were identified through 16S rDNA sequencing. (Tables 2 and 3) shows the identification of catfish bacterial isolates with the similarity percentage, length (base pairs), and Genbank accession number. A greater variety of bacterial species were identified from domestic pond catfish fillets than market catfish fillets. (Table 4) shows how effective FC vinegar was on the bacteria. Out of the twenty bacteria that were identified, eight were *Shewanella putrfaciens* with a zone of inhibition diameter measured at 10-15 mm. According to Ruiter, since acetate is the end product during the TMA-dependent respiration of specific spoilage bacteria such as *Shewanella* species, adding acetate to fish tissue can inhibit the TMAO-reduction by bacterial trimethylamine oxide reductases [31]. *Stenotrophomonas* and *Aeromonas* strains also exhibited a zone of inhibition diameter measured at 10-15 mm. *Enterobacteriaceae bacterium*/*Rahnella aquatilis and Pseudomonas fragi* showed a zone of inhibition diameter measured at 8-10 mm. The other *Pseudomonas* strains, *Pantoea* and *Plesiomonas* showed a zone of inhibition with 6-8 mm diameter.

Changes in Microbial Growth with Vinegar Treatments

Domestic catfish fillets with no vinegar treatment and treatments of 0.03, 0.05, 0.5, and 5% acetic acid dilutions of FC vinegar were stored at 4°C for 20 days. (Figure 1) illustrates changes in total bacteria enumerated every two days. The bacterial growth increased slightly less than the control for treatments with 0.03 and 0.05% acetic acid of vinegar and was largely reduced after 15 minutes of marinating in 5% acetic acid of the vinegar sample. There was a large reduction in bacterial growth for vinegar 0.5% and then remained constant in bacteria growth with little variation. These results were similar to a study by Bal'a and Marshall who evaluated the microbiological changes of catfish fillets following treatment of dipping 2% acetic acid [32]. He concluded that little microbial proliferation was observed. The shelf life of modified atmosphere packaged fillets can be prolonged by spraying with 10% acetic acid/acetate [33]. FC 5% maintained a large reduction in bacterial growth from day 0 to day 12 and remained at a constant of 0 CFU/g thereafter. Therefore, the addition of FC 0.5 and 5% prolongs the shelf life of domestic catfish fillets more than FC 0.03 and 0.05%.

Sensory evaluation of baked catfish fillets with vinegar treatments

Twenty-one panelists evaluated four sensory attributes; appearance, aroma, flavor, and texture for the four samples including the control using the Hedonic nine-point scale. The samples consisted of control and three treatments, FC 0.05, FC 0.5, and FC 5%. The least liked from the sensory evaluation were aroma and flavor in the catfish fillets marinated with FC 5% compared to the other samples. The aroma average for FC 5% was 3.71, "dislike moderately". The flavor average for FC 5% was 4, dislike slightly (Table 5). The aroma and the flavor showed the lowest results in the 5% marinade sample because of the strong aroma and flavor of the acid. One study used organic acids to prolong the shelf life of fresh salmon [34]. Salmon samples treated with acetic acid marinades showed significant acid odor and flavor. Minimal differences were obtained among the other samples (control, FC 0.05, and FC 0.5) on all four attributes with averages ranging from 5.19 to 6.43. The average ratings of appearance and texture ranged from 5 to 6.43. Appearance and texture were consistent in rating among all samples compared to aroma and flavor. The only color changed observed was on the fillet with FC 5% because of the vinegar's dark color. The more diluted vinegar samples did not cause a change in color on the fillet. According to Pons-Sanchez-Cascado et al. marinating involves changes in textural properties of fish [35]. In their study, anchovies (*Engraulis encrasicholus*) were immersed in a vinegar solution (20% acetic acid v/v) and vacuum-packed and resulted in a color change in the anchovies [35]. In a study of Schirmer et al. with organic acids on fresh salmon, no significant differences were found for hardness [34].

The statistical analysis was based on the Hedonic nine-point scale of four attributes per four samples. Values of $p<0.05$ were deemed significant. The most significantly different sample was FC 5% ($p=0.02$). Statistical analysis show that the attribute aroma of sample FC 5% was the most significant attribute with sample indicating the least liked of the panelists. Aroma was significantly different compared to appearance ($p=0.04$) and texture ($p=0.003$). Flavor of sample FC 5% was also significant among other samples and attributes ($p<0.05$). No significant differences were observed between the control and the vinegar treated samples with low acetic acid dilutions.

Conclusion

Vinegar applied as an antimicrobial agent in this study was shown to be effective in reducing spoilage bacteria. This was the only study that evaluated the inhibitory effect of acetic acid from vinegar on bacteria isolated from catfish. Fish and chip vinegar was the most effective on *Shewanella putrifaciens* isolated from catfish fillets from the market and local aquaculture pond. Vinegars were more effective on bacteria isolated from retail catfish fillets than local pond catfish fillets. Fish and chip vinegar containing 5% acetic acid would be appropriate in reducing bacteria but an acetic acid dilution of vinegar less than 5% appeals more to consumers in terms of aroma and flavor. Therefore, the most suitable acetic acid dilution of vinegar on catfish fillets would be 0.5% to prolong shelf life without diminishing the product sensory quality for consumers. The vinegar products can be applied as natural antimicrobial agents that can increase the safety, shelf life, and quality of fishery and other food products.

Acknowledgements

The authors would like to thank Dr. Gary Richards, USDA ARS, for providing his continuous support and assistance. This study was supported by EPSCoR RII CIBER Seed Grants.

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This article was originally published in a special issue, **Catfish Safety** handled by Editor(s). Dr. Christopher H Sommers, EUSDA's Eastern Regional Research Center, USA