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The Effect of Organic Acids on Compositional Characteristic of Protein and Lipid Recovered During Isoelectric Solubilization/Precipitation Processing of Silver Carp and the Effectiveness of these Organic Acids on the Reduction of Staphylococcus aureus

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The Effect of Organic Acids on Compositional Characteristic of Protein and Lipid Recovered During Isoelectric Solubilization/Precipitation Processing of Silver Carp and the Effectiveness of these Organic Acids on the Reduction of *Staphylococcus aureus*

Maryam Ronaghi

A thesis submitted to the
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2013

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ABSTRACT

The Effect of Organic Acids on Compositional Characteristic of Protein and Lipid Recovered During Isoelectric Solubilization/Precipitation Processing of Silver Carp and the Effectiveness of these Organic Acids on the Reduction of *Staphylococcus aureus*

Maryam Ronaghi

An effective method to recover protein, called ISP processing, uses extreme pH shifts to solubilize protein and then recover it by precipitation and centrifugation. The objective of this study was to determine the proximate composition of the recovered materials from ISP after processing with different acid concentrations and the bactericidal effectiveness of these acids in comparison with hydrochloric acid (HCl) and acetic acid against *Staphylococcus aureus* during isoelectric solubilization and precipitation (ISP) processing. Headed and gutted silver carp were homogenized and brought to the protein solubilization pH (2.5, 3.0, 11.5, or 12.5) using the acids (glacial L-lactic acid and formic acid (F&L) in sterile distilled water at a 1:1 ratio, glacial acetic acid or concentrated hydrochloric acid) or sodium hydroxide. Concentrations of F&L tested were 30%, 45%, 60%, 75%, 90% and 100% (v/v). Mixture was centrifuged to separate and remove lipid and insoluble fractions. The solubilized protein was brought to the isoelectric point (pH 5.5) with the addition of NaOH or acids and recovered by centrifugation. There were no differences in proximate composition (moisture, total fat, crude protein, ash) of the recovered protein fractions ($P > 0.05$), regardless of acid concentration, with the average protein concentration at 90.6%. Bactericidal effectiveness was tested on *S. aureus* at processing pH 11.5 and 12.5 using 30% formic and lactic combination and at processing pH 2.5, 3.0, 11.5 and 12.5 using HCl or acetic acid. Microbial analysis was performed on all fractions (lipid, protein, insoluble and water) and survivors were enumerated on Baird Parker and TSA media. Significant differences were observed between the selective and growth media ($P < 0.05$) at processing pH 11.5, while using F&L, indicating cell injury. However, no significant differences were observed between the selective and growth media ($P > 0.05$) while using HCl or acetic acid except for in the insoluble fractions. The greatest microbial reductions occurred at pH 12.5 using F&L and acetic acid with a mean log reduction of 2.61 and 2.49 CFU/g respectively in the protein fraction and a total log reduction of 2.47 and 2.45 CFU/g respectively. The results showed that a net-pasteurization effect did not occur (a 6-log reduction in microbial population) at any of the pH conditions and while ISP will significantly reduce *S. aureus*, further processing is required to achieve a net pasteurization effect.

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TABLE OF CONTENTS

<u>ABSTRACT</u>	<u>ii</u>
<u>ACKNOWLEDGEMENTS</u>	<u>iii</u>
<u>LIST OF TABLES</u>	<u>vi</u>
<u>LIST OF FIGURES</u>	<u>vii</u>
<u>CHAPTER I</u>	<u>1</u>
INTRODUCTION	1
REFERENCES	4
<u>CHAPTER II</u>	<u>6</u>
REVIEW OF LITERATURE	6
Effect of pH on protein recovery yield	7
Effect of ISP on compositional characteristics of recovered components	8
Effect of organic acids on protein and lipid recovery yield and proximate composition during ISP	9
<i>Staphylococcus aureus</i>	11
Effect of ISP processing on bacterial reduction	14
REFERENCES	17

CHAPTER III	21
INTRODUCTION	23
MATERIALS AND METHODS	26
RESULTS AND DISCUSSION	30
Part 1: Acid concentrations on proximate composition	30
Part 2: Bactericidal Effectiveness	32
REFERENCES	36
CHAPTER IV	42
INTRODUCTION	44
MATERIALS AND METHODS	46
RESULTS AND DISCUSSION	48
REFERENCES	53

LIST OF TABLES

CHAPTER III

Table 1. Proximate composition^a (percent, dry basis) of recovered lipids, proteins and insolubles from Silver carp after ISP processing using different formic and lactic acid concentrations in water at pH 11.5 **39**

Table 2. Reduction of *S. aureus* by ISP processing with 30% formic and lactic acid (1:1 ratio) in silver carp. Values were determined by subtraction of the log of the recovered cells within a fraction from the log of the total initial inoculation (average: 7.1 log CFU/g).). There were significant differences in recovery from **BP** and **TSA** ($P < 0.05$). **40**

CHAPTER IV

Table 1. Log reduction of *S. aureus* by ISP processing with acetic and hydrochloric acids in silver carp. Values were determined by subtraction of the log of recovered cells within a fraction from the log of the total initial inoculation (average: 7.10 log CFU/g). **56**

Table 2. Log reduction of *S. aureus* exposed to several pH shifts in different fractions of headed and gutted silver carp. Values were determined by subtraction of the log of recovered cells within a fraction from the log of the total initial inoculation (7.10 log CFU/g). Combined data from TSA and BP There were no significant differences in recovery between TSA and BP ($P > 0.05$). **58**

LIST OF FIGURES

CHAPTER III

Figure 1. Recovered *S. aureus* at pH 11.5 on BP and TSA after ISP processing. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$. **41**

Figure 2. Recovered *S. aureus* at pH 12.5 after ISP processing. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$. **41**

CHAPTER IV

Figure 1. Recovered *S.aureus* after ISP processing of silver carp at acidic processing pH levels. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$. **59**

Figure 2. Recovered *S.aureus* after ISP processing of silver carp at basic processing pH levels. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$. **60**

Chapter I

Introduction

The silver carp (*Hypophthalmichthys molitrix*) is a freshwater fish native to north and northeast Asia. In the early 1970s, these fish were brought to North America from China in an attempt to remove algae from aquaculture ponds (Conover et al., 2007). After escaping from these aquaculture ponds, they established reproducing populations in the Mississippi, Missouri, Ohio, and Illinois rivers (Chick et al., 2001). These fish are considered to be unsuitable for human consumption in the United States. However, due to their minimum growth requirements and rapid growth rate, commercial processing has become of interest. Due to the bony nature of silver carp, typical mechanical means are at removing their bones and recovering the meat. Therefore, an alternative method should be utilized to recover valuable protein and lipids from these fish (Taskaya et al., 2009).

Isoelectric solubilization and precipitation (ISP) is a method that utilizes extreme pH shifts (acidic or basic) to solubilize protein and separate lipid and insoluble fractions from animal tissue by centrifugation. The solubilized protein is recovered by returning the solution to its isoelectric point to allow the protein to precipitate and separate it from water by centrifuging a second time (Taskaya et al., 2009). Since this is a non-thermal process, it is important to determine the extent at which the process will reduce bacterial load in the recovered protein. The bactericidal effectiveness of the ISP protein recovery process is limited when hydrochloric acid (HCl) is used as the processing acid. Previous studies have demonstrated that while HCl is effective at significantly reducing *Escherichia coli* and *Listeria innocua* in ISP-recovered fish protein (Otto et al., 2011 a & b; Lansdowne et al., 2009 a & b); however, microbial reductions did not provide a

6-log reduction, which is the reduction amount needed to be considered a pasteurization effect (FDA, 2011).

Strong acids like HCl dissociate within the solution and outside the bacterial cell. Acid shock or acid stress can occur in low pH conditions when H⁺ ions cross the cell membrane and lower intracellular pH (Abee and Wouters, 1999). On the other hand, organic acids enter the cell in their undissociated form and dissociate once inside the cell. The bactericidal effectiveness of an acid is dependent on the dissociation of the acid within the bacterial cell. The bacterial cell must actively remove the free protons released by the acid and a high concentration of protons will cause cell death by ATP depletion (Jay et al., 2005).

The bactericidal effectiveness of organic acids (citric and acetic acid) compared to HCl have been demonstrated (Otto et al., 2011b). Greater reductions were observed in *L. innocua* populations when using citric acid or acetic acid compared to HCl when protein was solubilized at acidic conditions. Also, a net pasteurization effect (6-log reduction in microbial populations) was observed in all the recovered fractions when using acetic acid as the ISP processing acid at protein solubilization pH 3.0 (Otto et al., 2011b).

Staphylococcus aureus is a facultative anaerobic Gram-positive cocci bacterium that is mostly found in the nostrils and on the skin and hair of warm-blooded animals (Doyle et al., 2001). Most contaminations of food by *S.aureus* can be traced back to human carriers as well as contaminated equipment that were involved in the preparation process. These bacteria have the ability to tolerate a wide range of temperatures, pH environments, salt concentrations, and are able to survive for extended periods in a dry state (Doyle et al., 2001).

Food poisoning by *S.aureus* is a common cause of gastroenteritis which is not due to the ingestion of the live organism but rather is a result of the production of enterotoxin in the staphylococci-contaminated food (Doyle et al., 2001). Symptoms of food poisoning include nausea, violent vomiting, abdominal cramping, sweating, headache, prostration, diarrhea and sometimes a drop in body temperature. Occasional hospitalization is possible for elderly and infants, but the mortality rate is very low (Bore et al., 2007). It is possible that *Staphylococci* exist, at least in low numbers, in all or most animal origin food products or products that have been handled directly by humans and have not been heat processed properly (Bore et al., 2007).

It has been demonstrated that the combination of antibacterial agents have a stronger antibacterial effect in contrast with each one alone. Other studies have determined that the combination of organic acids may cause a synergistic antibacterial effect. Raftari and others (2009) tested the effects of different combinations of propionic, formic and lactic acid at a 1: 1 ratio and 1%, 1.5%, and 2% concentrations when sprayed onto beef carcasses, on *Staphylococcus aureus* and *E. coli*. They found the combination of lactic and formic acid had the most lethal effect on *S. aureus*. Therefore, the objectives of this thesis was to

- 1) Determine the most effective concentration of formic and lactic acid on lipid and protein recovery yield during isoelectric solubilization and precipitation of silver carp protein.
- 2) Determine the bactericidal effectiveness of these acids against *Staphylococcus aureus* during ISP processing.
- 3) Compare the effectiveness of hydrochloric acid to acetic acid on the reduction of *Staphylococcus aureus* during the ISP protein recovery process.

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Chapter II

Review of Literature

Silver carp (*Hypophthalmichthys molitrix*) are a species of freshwater cyprinid fish, which are a variety of Asian carp native to north and northeast Asia. Silver carp are not cultured for marketing in the USA because of their jumping habits and poor handling qualities during production. They were first brought to North America from China in the early 1970s to remove algae from aquaculture ponds (Conover et al., 2007). They escaped from the aquaculture ponds into the Mississippi river, and as a result established reproducing populations in the Mississippi, Missouri, Ohio, and Illinois rivers (Chick et al., 2001). They are considered to be unsuitable for human consumption in the USA, but because of their rapid growth rate and minimum growth requirement, commercial processing has become of interest. Due to the bony nature of their carcass, typical mechanical methods are not efficient for removing their pin bones. Therefore, an alternative method should be utilized to recover valuable protein and lipid from carp. Isoelectric solubilization and precipitation (ISP) processing is a recovery method that separates protein and lipid from carcasses using extremely high or low pH conditions. The protein is solubilized at the extreme pH and then recovered by precipitation at an optimum isoelectric point and centrifugation (Taskaya et al., 2009b). Lipids are recovered by centrifugation. In the traditional method of performing ISP, hydrochloric acid (HCl) is the acid of choice. However, some studies have shown organic acids to be more effective in bacterial reduction (Lansdowne et al., 2009 a & b; Otto et al., 2011a & b). Therefore, the effect of processing acids on protein and lipid recovery yield, as well as their bactericidal effectiveness, needs to be explored.

Effect of pH on protein recovery yield

Muscles in fish consist mainly of myofibril and sarcoplasmic proteins. The myofibrillar proteins are salt soluble proteins since they are not soluble at physiological pH values unless salt is present at a relatively high concentration ($> 0.3\text{M}$). However, these proteins can become soluble at low ionic strengths or very low or high pH levels (Stefansson and Hultin, 1994). During ISP, with the addition of acids and bases, IS values change, which impacts the solubility of proteins in solution. When these acids and bases dissociate in the solution, the conductivity, and therefore ionic strength increase accordingly. Chen and Jaczynski (2007a) have shown how changes in pH and IS affect the solubility of these muscle proteins. They found that water-insoluble proteins have a maximum precipitation at pH 5-6 and they are soluble at pH values higher than 6 and lower than 5. However, water soluble proteins approached 80% solubility at pH 5-5.5. Their data confirmed that solubility of water-soluble proteins isolated from trout decreased with increasing IS. Most proteins were recovered after solubilization with acid or base followed by precipitation. After applying nine different pH combinations, they found the best solubilization pH values, where 90% of proteins were recovered, were 2.5 or 13.0 followed by their precipitation at 5.5 (Chen and Jaczynski, 2007a). In a study by Kristinsson and others (2006) ISP processing on Atlantic croaker resulted in better protein recovery yields at acidic processing pH values rather than basic. This difference may be explained by the solubility behavior of sarcoplasmic proteins at pH 5.5. According to Kristinsson and Hultin (2004), ISP processing at basic pH values lead to less protein denaturation and protein coaggregation at pH 5.5. This may lead to less overall protein recovery at basic processing pH values. In comparison, a study by Taskaya et al. (2009b) showed that a higher protein and lipid recovery yield (94-97% and 88-89%) was achieved by using basic pH values for protein solubilization compared to acidic pH

values (89-90% and 94-97%). Taskaya and others (2009b) determined their protein concentration by Kjeldahl assay while Kistinsson and Hultin (2004) used the Biuret method. Therefore, the difference in recovery yields may have been due to the difference in the methods used to determine protein concentration. In addition, carp is a freshwater species unlike Atlantic croaker which is a saltwater fish. Therefore, the lower protein recovery yield in croaker may have been due to inherent salinity (i.e., increased IS). Therefore, aside from pH values, the source of protein may also affect protein recovery yields during ISP processing.

Effect of ISP on Compositional Characteristics of Recovered Components

Several studies have demonstrated the effect of ISP on the proximate composition (including ash, moisture, lipid and crude protein) of the recovered components (protein, lipid and insolubles). During ISP, whole animals (including exoskeleton, bone, appendages and other inedible impurities) are used as the starting material (Chen et al., 2009). These impurities typically have high mineral contents; therefore, ash content is used as an indicator of how well they are removed from the recovered components (Chen et al., 2009). Basic treatments tend to yield lower ash content in the recovered protein and lipid fractions of whole gutted silver carp compared to acidic (Taskaya et al., 2009 a & b). Additionally, higher ($P < 0.05$) ash contents were observed in the insoluble components after using basic processing pH values compared to acidic. Similar ash contents were reported in the protein and insoluble fractions recovered from whole krill (Chen and Jaczynski, 2007b), rainbow trout (Chen et al., 2007) and headed and gutted silver carp (Paker et al., 2013).

It is desirable to have less fat content in the proteins due to the possibility that they will become rancid. In a study Taskaya and others (2009 a & b), basic treatments resulted in greater

($P < 0.05$) fat removal from the recovered protein from whole gutted silver carp compared to acidic treatments. Similar results have been reported for channel catfish (Kristinsson et al., 2005), herring (Undeland et al., 2002), Atlantic croaker (Kristinsson et al., 2006), Antarctic krill (Chen and Jaczynski, 2007b) and rainbow trout (Chen and Jaczynski, 2007a). When processing oil to obtain a soapstock, free fatty acids are more readily removed with alkaline processing than acidic treatment (Nawar et al., 1996), which may in part explain the greater removal of fat from protein during basic treatments.

During ISP, the recovered insoluble fraction may contain some fat and crude protein. Taskaya and others (2009a) observed that the proteins recovered in the insoluble fraction did not solubilize with pH alteration, suggesting they were most likely non-muscle nitrogenous compounds. The lipids found in the insoluble fraction were likely phospholipids that can bond with water dipoles and hydrophilic parts of proteins and as a result were not separated with centrifugation (Taskaya et al., 2009a).

Effect of Organic Acids on Protein and Lipid Recovery Yield and Proximate Composition During ISP

The protein recovery yield is essential to determine the economic feasibility of a new technology. Isoelectric solubilization/precipitation can also recover fish oil which may offer human health benefits. Therefore, determination of lipid recovery yield may be helpful in understanding the economic feasibility of this method (Taskaya et al., 2009b; Gehring et al., 2011).

Different protein yields have been reported at different processing pH values while using HCl. Protein recovery yields of ISP reported in the literature range between 42% and 90% (Chen and Jaczynski, 2007b; Chen et al., 2009; Kristinsson and Liang, 2006; Taskaya et al., 2009b). During ISP processing of rainbow trout (Chen and Jaczynski, 2007b), whole Antarctic krill (Chen et al., 2009) and Atlantic croaker (Kristinsson and Liang, 2006), higher protein recovery yields were reported at acidic processing pHs. However, Taskaya and others (2009b) reported that higher ($P<0.05$) protein recovery yields were observed at basic processing pHs. Taskaya and others (2009b) used Kjeldahl assay to determine crude protein content while Kristinsson and Liang (2006) used the Biuret method which may have led to the differences in protein recovery yields. Additionally, different species of fish, centrifugation force used during ISP and a relative concentration of water-soluble sarcoplasmic protein may have resulted in different outcomes. Sarcoplasmic proteins are only partly recovered during ISP (Chen and Jaczynski, 2007b).

In a study by Taskaya and others (2009b), basic solubilization during ISP processing of whole gutted silver carp, resulted in higher ($P<0.05$) lipid recovery yields compared to acidic pH values. The fat recovery yields indicated that ISP processing while using HCl as the processing acid, allowed efficient recovery of carp fat and may be an added benefit for fish processors.

In a study by Paker and others (2013), organic acids were used as the processing acid during ISP processing. They studied the effects of acetic acid and a 30% formic and lactic acid combination (F&L) on total protein and fat recovery yields, proximate composition and mineral composition on recovered fractions. They found the highest protein and lipid recovery yield was achieved at acidic solubilization pH 2.5 when using acetic acid and the lowest protein and lipid recovery yield was at acidic solubilization pH values when using F&L (Paker et al., 2013).

As mentioned earlier, it has been demonstrated that basic processing pH values were most effective at removing impurities from recovered protein and lipid contents and had the lowest ash content. However, in the study by Paker and others (2013), organic acids were not as successful as HCl at separating protein, lipid and insoluble fractions during ISP. This may be due to the difference between pKa value of these acids. The amount of acids present in their dissociated form in the solution is dependent on their pKa. The pKa value of an acid is the pH where the acid is 50% in the dissociated form and 50% in the undissociated form. In this study acetic acid, lactic and formic acid have pKa values over 3.7 which means that they will not be 100% in their dissociated form during ISP processing at acidic pH values. This may explain why acidic processing pH levels with the use of organic acids had a lower protein concentration in their protein fractions. Strong acids like HCl have low pKa values (-6) which is why using these acids during ISP processing will result in a higher protein concentration in the protein fractions at acidic processing pH values. Basic processing pH values while using organic acids resulted in a higher protein concentration compared to acidic pH values. However, the protein concentrations in the recovered proteins were not as high as those reported when HCl was used (Paker et al., 2013).

Staphylococcus aureus

Staphylococcus aureus is a facultative anaerobic Gram-positive cocci bacterium. *S. aureus* is mostly found in the nostrils and on the skin and hair of warm-blooded animals. Most contaminations of food by *S.aureus* can be traced back to human carriers as well as contaminated equipment that were involved in the preparation process. These bacteria have the ability to tolerate a wide range of temperatures (7° to 48.5°C with an optimum of 30 to 37°C), pH (4.2 to

9.3, with an optimum of 7 to 7.5) and sodium chloride concentrations (up to 15% NaCl) and survive for extended periods in a dry state (Doyle et al., 2001).

According to the Center for Disease Control and Prevention, *Staphylococcus aureus*, with an estimate of 241,148 illnesses in 2011, is in the top five of pathogens that result in domestically acquired foodborne illnesses (CDC, 2013). Food poisoning by *S. aureus* is not due to the ingestion of the live organism but rather is a result of the production of enterotoxin in the staphylococci-contaminated food (Doyle et al., 2001). Vegetative cells are commonly found on the skin and in the nose of 25% of healthy people and they do not cause an illness for their carriers. The numbers of bacteria per square inch are higher in moist areas (10^3 - 10^6) rather than dry (10 - 10^3) (Kloos et al., 1994). The production of toxin by *S. aureus* is favored by optimal conditions; however, *Staphylococci* can grow under conditions that do not favor enterotoxin production (James et al., 2005). Toxin is produced throughout the logarithmic phase of growth or during the transition between the exponential and stationary phases. This bacterium is a poor competitor in complex bacterial populations which is why the greatest risk of food poisoning is when the initial micro flora is inhibited or destroyed (Bore et al., 2007). Symptoms of food poisoning by *S. aureus* start after 2-8 h of toxin consumption and typically resolve within 24-48 h. These symptoms include nausea, violent vomiting, abdominal cramping, sweating, headache, prostration, diarrhea and sometimes a drop in body temperature. Occasional hospitalization is possible for elderly and infants, but the mortality rate is very low (Bore et al., 2007).

A common strategy to reduce bacterial growth is by the use of acid and a reduction in pH. However, *Staphylococcus aureus* has a high tolerance for acids and may use a number of mechanisms to cope with a drop in pH levels. Generally, bacteria use proton pumps, which pump

protons out of the cell to keep the internal pH level at an acceptable level. Another coping mechanism in bacteria is to increase the alkaline compounds inside the cell to counteract the acidification of the cytoplasm. Some bacteria can also form biofilms which may affect their acid resistance. According to Rode et al. (2010), *S. aureus* relies on ammonia production and the removal of acid groups to increase the pH in the medium when exposed to organic acid stress at low pH at exponential growth.

It has been found that organic acids and their salts have the ability to extend the shelf-life of refrigerated meats, poultry and fish by inhibiting pathogenic and spoilage bacterial growth (Ratanatriwong et al., 2009). Common organic acids that are used as additives or are naturally occurring in foods include acetic, lactic, citric, sorbic, benzoic, propionic, methyl and propyl esters of parahydroxybenzoic acid. According to Eifert et al. (1997), acetic acid inhibits bacterial growth by neutralizing the electrochemical potential of bacterial cell membranes and lowering their intracellular pH. Acetic acid has a stronger antimicrobial activity compared to lactic acid which in part may be due to its higher pKa. When a mixture of acid is present, it is likely that lactic acid only contributes to a reduction in pH. However, it has been observed that lactic acid also has the ability to permeate membranes which will enhance the antimicrobial activity of other substances (Salminen et al., 2004). Strong acids (i.e. hydrochloric acid) have a lower pKa compared to weak, organic acids which means strong acids are in their dissociated form in most pH conditions. Undissociated acids have a greater effect on microbial growth which is why organic acids have a better effect on microbial growth (Eifert et al., 1997).

Effect of ISP Processing on Bacterial Reduction

In the traditional method of ISP processing, HCl is the acid of choice. It is effective at reducing *Escherichia coli* and *Listeria innocua*, however, a net pasteurization effect (a 6-log reduction in microbial population) was not reported (Lansdowne et al., 2009 a & b). The best reductions of *L. innocua* occurred when protein was solubilized at pH 2.0 with a 3.1 log reduction; on the other hand, the best reductions of *E. coli* occurred when protein was solubilized pH 12.5, with a 4.4 log reduction (Lansdowne et al., 2009 a & b).

Unlike strong acids that completely dissociate in solutions, organic acids dissociate once inside the bacterial cell which creates a greater drop in the intracytoplasm pH. Organic acids have the ability to diffuse through the bacterial cell membrane and dissociate once inside the cell. This creates a more toxic environment for bacteria and therefore, organic acids have a better effect on microbial load reduction. A study by Otto et al. (2011b) evaluated the effect of acetic acid and citric acid on the reduction of *Listeria innocua* during ISP processing of rainbow trout. Significant reductions in microbial population loads were observed regardless of acid type or processing pH. The greatest reduction was observed when protein was solubilized at pH 3.0 with acetic acid which resulted in a 5.88 log reduction in the combined components. When *L. innocua* was treated with HCl at pH 3.0, only a 0.7 log reduction was observed in the combined components. Acetic acid was more effective than HCl in microbial load reduction compared to strong inorganic acids like HCl.

When the effect of using citric and acetic acid as the process acid during ISP processing on the reduction of *Listeria monocytogenes* was evaluated on rainbow trout there were significant microbial reductions regardless of solubilization pH (Otto et al., 2011a). However, the greatest

reductions were when protein was solubilized at pH 3.0 using acetic acid, with a mean log reduction of 3.03 in the combined components. *L. monocytogenes* has the ability to resist highly acidic treatments by upregulating certain proteins that alter the structures of the cell's membrane and therefore, increase the cell's ability to maintain intracellular pH. σ^B is a sigma factor (protein subunits that enable binding of bacterial RNA polymerase to specific gene promoters) that is seen in Gram-positive bacteria (including *Staphylococcus aureus*) which contributes to acid tolerance exhibited by *Listeria* (Raengpradub et al., 2008). The genes that are regulated by this sigma factor are unique to each species. *L. monocytogenes* and *L. innocua* share 49 genes that are regulated by σ^B . However, *L. monocytogenes* has over 140 genes that are believed to be affected by this sigma factor. As a pathogen, *L. monocytogenes* has the ability to survive in a low pH like the gastric fluid. Therefore, it is believed that the σ^B stress response has adapted to this pathogen's lifestyle and may be the reason for the difference in survival between the 2 species (Ferreira et al., 2003; Raengpradub et al., 2008). The same sigma factor, σ^B , is also present in *Staphylococcus aureus* and it contributes to acid tolerance in these bacteria.

Using a combination of organic acids might have a stronger bactericidal effect during ISP. This theory was further investigated by Laury et al. (2009) where they dipped a chicken inoculated with *Salmonella* in a mixture of lactic and citric acid for 5s which resulted in a significant reduction of 2.5 logs. When acetic, lactic, propionic and formic acid were combined 1:1 at 1%, 1.5% and 2% concentrations and sprayed onto beef carcasses, significant reductions of *Staphylococcus aureus* were achieved (Raftari et al., 2009). It was also shown that the most lethal combination was with 1:1 lactic and formic acids at all concentrations (Raftari et al., 2009). These findings demonstrate the bactericidal potential of using organic acids and combinations of organic acids in the ISP process.

Summary

Staphylococcus aureus has a high tolerance for high temperatures, acidic conditions and high sodium chloride content. In most cases contamination with these bacteria is due to contaminated equipment or human carriers that handled the food. Organic acids have been shown to be more effective at reducing bacteria during ISP processing. Therefore the purpose of this study was to determine the most effective formic and lactic acid combination on lipid and protein recovery yield during ISP processing of silver carp. This formic and lactic acid combination was used to test the effectiveness of ISP processing to reduce *Staphylococcus aureus* in silver carp and results were compared to microbial recovery when HCl and acetic acid were used.

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Chapter III

Isoelectric Solubilization and Precipitation of Silver Carp Protein with Increasing Formic and Lactic Acid Concentrations and their Bactericidal Effectiveness on *Staphylococcus aureus*

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S. aureus After Protein Recovery Process (...)

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Abstract

The objective of this study was to determine the effect of different organic acid concentrations on protein concentration and the bactericidal effectiveness of these acids against *Staphylococcus aureus* during isoelectric solubilization and precipitation (ISP) processing. Headed and gutted silver carp were homogenized and brought to the protein solubilization pH (11.5) with the addition of NaOH. Mixture was centrifuged to separate and remove lipid and insoluble fractions. The solubilized protein was brought to the isoelectric point (pH 5.5) using glacial L-lactic acid and formic acid (F&L) in sterile distilled water at a 1:1 ratio and recovered by centrifugation. Concentrations tested were 30%, 45%, 60%, 75%, 90% and 100% (v/v). There were no differences in the concentration of crude protein in the recovered protein fractions ($P > 0.05$), regardless of acid concentration, with the average protein concentration at 90.6%. Bactericidal effectiveness was tested on *S. aureus* at processing pH 11.5 and 12.5 using 30% formic and lactic acid combination. Microbial analysis was performed on all fractions (lipid, protein, insoluble and water) and survivors were enumerated on Baird Parker and TSA media. Significant differences were observed between the selective and growth media ($P < 0.05$) at processing pH 11.5, indicating cell injury. The greatest microbial reductions occurred at pH 12.5 with a mean log reduction of 2.61 CFU/g in the protein fraction and a total log reduction of 2.47 CFU/g. The results showed that a net-pasteurization effect did not occur (a 6-log reduction in microbial population) at any of the pH conditions.

KEYWORDS: *Staphylococcus aureus*, fish, formic acid, protein recovery, organic acid

Introduction

The silver carp (*Hypophthalmichthys molitrix*) is a freshwater cyprinid fish native to north and northeast Asia. Silver carp were introduced to North America from China in the early 1970s to remove algae from aquaculture ponds (Conover et al., 2007). They escaped from the aquaculture ponds into the Mississippi River, and as a result established reproducing populations in the Mississippi, Missouri, Ohio, and Illinois rivers (Chick et al., 2001). Silver carp are not typically harvested for human consumption in the United States because standard fish processing methods are not efficient at removing pin bones from their boney carcasses; however, their rapid growth rate and minimum growth requirements make silver carp a resource of high quality protein and lipids that is not currently being utilized.

Isoelectric solubilization and precipitation (ISP) method is a recovery process that uses extremely high or low pH conditions to solubilize protein and separate lipid in animal tissue. The protein is then precipitated at the isoelectric point of the protein and recovered by centrifugation (Taskaya et al., 2009b). ISP process recovery yields are species specific and dependent on processing parameters such as pH of protein solubilization, pH of protein precipitation, type of processing acid/base used, etc (Chen and Jaczynski, 2007b; Chen et al., 2009; Paker et al., 2013; Taskaya et al., 2009b).

The purity of the recovered protein is an important indicator of ISP effectiveness; proximate composition is used to determine the make-up of the recovered protein fraction. The concentration of protein in the “recovered protein” fraction reported in the literature ranges from 54% to 95% for ISP-recovered protein (Chen and Jaczynski, 2007b; Paker et al., 2013; Taskaya et al., 2009 a & b). Protein concentration in the recovered protein fractions were best when protein

was solubilized in basic conditions for Atlantic croaker (Kristinsson and Liang, 2006) and silver carp (Paker et al., 2013; Taskaya et al., 2009 a & b).

Traditionally, hydrochloric acid is used as the processing acid with the ISP protein recovery method. Paker and others (2013) studied the effectiveness of acetic acid or a 30% formic and lactic acid combination (F&L) as the processing acid to recover protein from silver carp. When protein was solubilized under basic conditions, protein concentrations were comparable studies where HCl was used as the processing acid. Although proximate analysis revealed that basic solubilization conditions were more effective at removing impurities, the recovered fractions when HCl was used as the processing acid were more pure (Paker et al., 2013; Taskaya et al., 2009b). The bactericidal effectiveness of the ISP protein recovery process is limited when hydrochloric acid (HCl) is used as the processing acid to reduce of *Listeria innocua* in protein solubilized under acidic conditions and *Escherichia coli* in protein solubilized under basic solubilization conditions (Lansdowne et al. 2009 a & b). Unlike strong acids that completely dissociate in solutions, organic acids dissociate once inside the bacterial cell which creates a greater drop in the intracytoplasmic pH (Jay et al., 2005). Organic acids have the ability to diffuse through the bacterial cell membrane and dissociate once inside the cell. This creates a more toxic environment for bacteria and therefore, organic acids have a better effect on microbial load reduction (Eifert et al., 1997). Otto et al. (2011b) evaluated the effect of acetic acid and citric acid on the reduction of *Listeria innocua* during ISP processing of rainbow trout and reported higher reductions in microbial population loads compared to HCl.

Combining organic acids may create a synergistic antibacterial effect on bacteria. This may be due to an increase of protons ions or an increase in the undisociated form of these acids in an aqueous environment when they are combined as opposed to when they are used alone

(Malicki et al., 2004). Raftari and co-workers (2009) further investigated this effect by using different organic acids combinations at different concentrations as a spray, to treat *Staphylococcus aureus* on meat carcasses. They found significant reductions in microbial populations especially when formic and lactic acids were combined at a 1:1 ratio. Therefore, the objectives of this study were to determine 1) the most effective concentration of formic and lactic acid to maximize protein concentration during isoelectric solubilization and precipitation of silver carp protein, and 2) the bactericidal effectiveness of these acids against *Staphylococcus aureus* during ISP processing.

Materials and Methods

Part 1: Acid concentrations on proximate composition and recovery yields

Sample preparation. Whole, gutted silver carp (*Hypophthalmichthys molitrix*) was purchased (Fin International LLC., New Orleans, LA), shipped overnight on ice, headed and dipped for 10 sec in a 50 ppm bleach solution. The sanitized fish was ground into a thick paste in a food processor (BIRO, Marblehead, Ohio, model 12) which was sanitized using a 70% alcohol solution and UV light for 15min. The paste was divided into freezer bags and stored at -80°C (Lansdowne et al., 2009).

Isoelectric solubilization and precipitation. Approximately 255 g or 155 g (for bactericidal effectiveness study) of frozen carp was thawed at 4° C for 24 h. Defrosted fish was homogenized (Omni General Lab Homogenizer-115) with distilled, deionized water (4°C) at 1:6 (w/v) ratio, 10 N sodium hydroxide (NaOH) was added until the pH was 11.5 or 12.5 (for bactericidal effectiveness study), and the mixture was homogenized for an additional 10 min. Temperature was maintained at 4° C by submerging the beaker of paste in an ice bath to reduce the activity of carp endogenous proteases. The solution was transferred into centrifuge bottles and centrifuged at $10,000 \times G$ for 10 min at 4° C to separate the lipids and the insoluble portions (scales, bones, skin, etc.) from the protein. The supernatant, which includes solubilized protein, was poured through a cheese cloth to recover the lipid portion while the insoluble portions remained at the bottom of the centrifuge bottle. The supernatant solution with solubilized protein was brought to the isoelectric point (pH 5.5) using a combination of glacial L-lactic acid (85%, EMD Chemicals, Netherlands) and formic acid (88%, Mallinckrodt Chemicals, Netherlands) (F&L) in sterile distilled water at a 1:1 ratio. Concentrations tested were 30%, 45%, 60%, 75%, 90% and 100%

(v/v). After adjusting the pH, the mixture was homogenized for an additional 10 min to allow for protein precipitation. The precipitated proteins were centrifuged for a second time at 10,000 ×G for 10 min at 4 °C. Once again, the supernatant was poured through a sterilized cheese cloth and the protein remained at the bottom of the centrifuge bottle. All components (protein, lipid, water and insoluble components) were stored at -80°C until transfer to a freeze dryer (VirTis Genesis, SP Scientific, Gardiner, NY) until the probes read above 22°C, to indicate that the samples were mostly dry. Samples were stored at 4°C until further analyses were conducted.

Proximate Analysis. Proximate composition (moisture, crude protein, fat and ash) was determined on initial fish paste and the ISP-recovered components (lipid, protein and insoluble components) for all F&L concentrations according to the Association of Official Analytical Chemists (1995). Moisture content was measured by evenly spreading a sample on an aluminum plate and oven-drying at 105°C for 24 hr. Fat and crude protein content was determined by the Soxhlet extraction method and Kjeldahl assay, respectively. Ash content was determined by burning samples in a muffle furnace at 550° C for 24 h.

Part 2: Bactericidal effectiveness

***Staphylococcus* strain and inoculum preparation.** Bactericidal effectiveness was tested on *S. aureus* at ISP protein solubilization pH 11.5 and 12.5 using 30% formic and lactic combination. *S. aureus* lab strain (ATCC 25923) was revived in a 250 ml flask containing 100 ml sterile brain heart infusion media (BHI; all media were from Difco, Becton Dickinson, Sparks, MD unless otherwise stated) and incubated at 37°C for 18 to 24 h at 50 rpm in a rotary incubator (Barnstead lab-line, model 305, Melrose park, IL). The culture was transferred once by loop to a 250 ml flask

containing 100ml BHI and again incubated at 37°C for 24 h at 50 rpm. Cultures (24 h) were spread onto slants of trypticase soy agar (TSA) to create working stocks. Slants were incubated at 37°C for 18 to 24 h and then maintained for up to four weeks at 4°C.

Cultures were transferred twice by loop inocula from slants into 100 ml sterile BHI flasks and incubated at 37°C for 24 h in a rotary incubator at 50 rpm. After second 24-hour incubation period, cultures were transferred to sterile centrifuge bottles and centrifuged at 10,000 x G for 10 min at 4°C (Otto et al., 2011 a & b). Supernatant was removed and the remaining unwashed pellet was used to inoculate 155 g defrosted fish paste.

Inoculation of fish paste. Defrosted fish paste was transferred to an autoclaved ceramic dish and the *S. aureus* pellet was mixed into the paste with an autoclaved spatula. The inoculated fish paste was transferred to an autoclaved beaker for ISP processing. ISP processing was conducted as described above using 30% F&L at protein solubilization pH 11.5 and 12.5. Recovered fractions (protein, lipid, insoluble and water) were tested immediately for microbial survival.

Microbial analysis. Microbial analysis was performed on non-inoculated fish paste (background), inoculated fish (initial), and each fraction (lipid, protein, insoluble and water) recovered by ISP using 30% F&L as the processing acid. For each component, a 1 g sample was placed in a sterile stomacher bag, mixed with 9 ml sterile peptone buffer and hand pummeled for 2 min. Each constituent was tenfold serially diluted and 0.1 ml was spread plated onto TSA (non-selective) and Baird Parker (BP) medium (selective) plates and incubated at 37°C for 24 h. The detection limit for non-inoculated fish paste was $<10^3$ CFU/g, $<10^2$ CFU/g for lipid, protein and insoluble fractions, and <10 CFU/g for the process water (Otto et al., 2011 a & b). Total log

reduction was calculated by subtracting the summation of survivors in all fractions from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$ (Otto et al., 2011 a & b; Lansdowne et al., 2009a & b).

Statistical Analysis. The study was replicated three times for each F&L concentration. All proximate analyses were conducted in triplicate and reported as the mean value ($\pm$ SD) and expressed as g kg^{-1} . For the bactericidal effectiveness study, three replicated experiments were performed for each pH value. Microbial counts (CFU/g) were converted into logarithmic units and recovered cells were expressed as $\log_{10}$ CFU/g. A completely random design (CRD) was used (Steel et al., 1980) and data were analyzed by analysis of variance (ANOVA). Significance was determined at 0.05 probability level and Turkey's honestly significant differences test was used to determine differences in means. All statistical analyses of data were performed using JMP 10 (SAS Inst., Cary, N.C., USA).

Results and Discussion

Part 1: Acid concentrations on proximate composition

Proximate composition

The proximate composition of recovered lipids, proteins and insolubles from headed and gutted silver carp after ISP processing while using different concentrations of formic and lactic acid at pH 11.5 are shown in table 1.

No differences ($P>0.05$) were observed between the concentrations of crude protein or lipid (dry basis) in the recovered protein and lipid fractions, respectively at different acid concentrations. However, the concentration of protein was higher in the recovered proteins ($P<0.05$) in comparison to the initial fish at all acid concentrations. Protein and lipid concentrations ranged from 88-93% and 85-94% in the recovered protein and lipid fractions, respectively. Taskaya and others (2009 b) reported higher values of protein concentrations at 93% in the protein fraction and lipid concentrations at 94% in the lipid fraction when HCl was used as the processing acid at pH 11.5. This may be due to the fact that unlike strong acids, like HCl, organic acids do not dissociate completely in aqueous solutions. Organic acids, like formic and lactic acids, have different dissociation constants depending on the pH of the solution (Gehring et al., 2009). The amount of the available form of dissociated acid is in direct correlation to its ability to separate various fractions (protein, lipid, and insoluble) during the process of ISP. For example, when HCl was used as the processing acid at protein solubilization pH 11.5, the amount of protein and lipids recovered with the insoluble fraction were 44 and 19%, respectively (Taskaya et al., 2009b). Compared to our findings, the amount of protein and lipids recovered with the insoluble fraction that ranged from 55-64% and 18-36%, respectively. It is possible that the proteins found in the insoluble fractions were non-muscle nitrogenous

compounds which did not solubilize with pH alteration (Taskaya et al., 2009b). It is also possible that the lipids found in the insoluble fraction were phospholipids that were bound with water dipoles and the hydrophilic parts of proteins and as a result were not separated with centrifugation (Taskaya et al., 2009b). On the other hand, HCl dissociates completely in solution so it is possible that proteins were better separated from the other fractions. This would result in an increase in solubility of protein and separation of lipid, and therefore, increasing their concentration in their appropriate fractions. Changing the concentration of acid in solution will not affect the dissociation constant, which might explain why when the concentration of F&L increased there was no improvement in protein recovery.

Headed and gutted silver carp contains a high amount of impurities (skin, scales, bones, and so on) that need to be removed from the recovered protein and lipid components. Ash content is used as an indicator of how well these impurities were removed at each acid concentration. The average ash content (dry basis) in the recovered protein fraction was 1.99. Taskaya and others (2009b) reported an average ash concentration of 3.8% in the protein fraction when protein was solubilized at pH 11.5 using HCL, which is consistent with our results. These results imply that using F&L as the processing acid at solubilization pH 11.5 will remove impurities at least as effectively as when HCl is used as the processing acid. The concentration of ash in the lipid portion ranged from 0.31-1.76 with the 30% F&L solution having the greatest ash concentration and the least amount in the 100% F&L solution ($P < 0.05$), implying that the 100% acid concentration was the most effective at removing impurities from the recovered lipids.

Proximate composition of the recovered lipid fraction indicated that the lipid fraction recovered was very pure. The concentration of lipid in the recovered lipid fraction ranged from 89.45 – 91.95%; acid concentration did not affect lipid concentration of the recovered fraction (P

>0.05) since lipids were recovered before acids were added. However, Paker and others (2013) reported much lower lipid concentrations, at 60%. These differences were likely due to the high moisture content of the recovered lipid fraction. On the other hand, Taskaya and others (2009 a&b) reported lipid concentration at 94% which were consistent with the values reported in the present study.

Part 2: Bactericidal Effectiveness

Staphylococcus aureus

The average background flora of the inoculated fish was 3.4×10^3 CFU/g on TSA. There was no growth of *Staphylococcus* detected ($<10^2$ CFU/g) on Baird Parker. Significant differences ($P < 0.05$) were observed in recovery of cells on selective media (BP) in comparison with growth media (TSA) in the recovered protein, lipid and insoluble fractions at pH 11.5, suggesting that *S. aureus* cells were significantly injured when exposed to these conditions. The greatest microbial reductions occurred at pH 12.5 with a mean log reduction of 2.61 CFU/g in the protein fraction ($P > 0.05$) and a total log reduction of 2.47 CFU/g ($P < 0.05$). According to the U.S. Food and Drug Administration, pasteurization is defined as a 6-log reduction in microbial populations. However, a net pasteurization effect was not observed at any of the pH conditions. The highest number of recovered cells at pH 12.5 was observed in the protein fraction and at pH 11.5 the insoluble fraction contained the highest number of recovered cells. The lowest number of cells was recovered from the water fraction. Centrifugation is most likely responsible for the low number of cells in the recovered water fraction (Lansdowne et al., 2009a).

A common strategy to reduce bacterial growth is by the use of acids and a reduction in pH. However, *Staphylococcus aureus* has a high tolerance for acids and may use a number of

mechanisms to cope with a drop in pH levels. Generally, bacteria use proton pumps, which pump protons out of the cell to keep the internal pH level at an acceptable level. Another coping mechanism in bacteria is to increase the alkaline compounds inside the cell to counteract the acidification of the cytoplasm. Some bacteria can also form biofilms which may affect their acid resistance. According to Rode et al. (2010), *S. aureus* relies on ammonia production and the removal of acid groups to increase the pH in the medium when exposed to organic acid stress at low pH at exponential growth.

Unlike strong acids that completely dissociate in solutions, organic acids dissociate once inside the bacterial cell which creates a greater drop in the intracytoplasm pH. Organic acids have the ability to diffuse through the bacterial cell membrane and dissociate once inside the cell. This creates a more toxic environment for bacteria and therefore, organic acids have a better effect on microbial load reduction (Jay et al., 2005). When HCl was used during ISP processing of rainbow trout, significant reduction were observed in *Escherichia coli* and *Listeria innocua* ($P < 0.05$) in the recovered protein fraction (Lansdowne et al., 2009a & b). However a net pasteurization effect did not occur at any of the processing pHs. When Otto and others (2011b) used citric and acetic as the ISP processing acid for protein recovery in rainbow trout, they reported significant reductions ($P < 0.05$) of *L. innocua* regardless of pH or acid type. A 6.41 log CFU/g reduction was seen in the recovered protein fraction at processing pH 3.0 with acetic acid, compared to a 1.11 log CFU/g reduction when HCl was used as the processing acid (Lansdowne et al., 2009b). However, when the same organic acids and processing pHs were utilized during ISP processing of rainbow trout inoculated with *L. monocytogenes*, only a 3.53 CFU/g reduction occurred in the protein fraction (Otto et al., 2011a).

Using a combination of organic acids might have a stronger bactericidal effect during ISP. This theory was further investigated by Laury et al. (2009) where they dipped a chicken inoculated with *Salmonella* in a mixture of lactic and citric acid for 5 sec which resulted in a 2.5 log reduction of microbes. When acetic, lactic, propionic and formic acid were combined 1:1 at 1%, 1.5% and 2% concentrations and sprayed onto beef carcasses, significant reductions of *Staphylococcus aureus* were achieved (Raftari et al., 2009). It was also shown that the most lethal combination was with 1:1 lactic and formic acids at all concentrations (Raftari et al., 2009). These findings demonstrate the bactericidal potential of using organic acids and combinations of organic acids in the ISP process.

σ^B is a sigma factor (protein subunits that enable binding of bacterial RNA polymerase to specific gene promoters) that is seen in Gram-positive bacteria (including *Staphylococcus aureus*) which contributes to acid tolerance as seen in *Listeria* (Raengpradub et al., 2008). As a pathogen, *L.monocytogenes* has the ability to survive in a low pH like the gastric fluid. Therefore, it is believed that the σ^B stress response has adapted to this pathogen's lifestyle and may be the reason for the difference in survival between the 2 species (Ferreira et al., 2003; Raengpradub et al., 2008). The same sigma factor, σ^B , is also present in *Staphylococcus aureus* and it contributes to acid tolerance in these bacteria. This may explain the low number of log reductions of these bacteria during ISP processing of formic and lactic acid combination.

Conclusion

This study demonstrated that F&L at different concentrations (30%-100%) have the same effect on proximate composition (except for ash in lipid components) of recovered protein.

Therefore, 30% was the selected concentration to determine the bactericidal effectiveness of F&L on *S. aureus*. There were significant differences ($P < 0.05$) observed between TSA and BP media at pH 11.5 suggesting cell injury. There was a higher reduction ($P < 0.05$) in cells at pH 12.5, however, a net-pasteurization effect (6-log reduction in microbial populations) was not observed at any of the processing pHs. *Staphylococcus aureus* have a high tolerance for acidic conditions and have developed coping mechanisms. Therefore, future studies focusing on creating a hurdle effect with acid and other components, may be able to obtain a net-pasteurization effect after ISP processing.

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Table 1. Proximate composition ^a (percent, dry basis) of recovered lipids, proteins and insolubles from Silver carp after ISP processing using different formic and lactic acid concentrations in water at pH 11.5

Recovered Proteins			
Acid Concentration	Lipid	Protein	Ash
30%	4.12±1.03b	89.31±1.16	1.92±0.02ab
45%	2.82±0.62b	89.90±1.06	2.40±0.26a
60%	3.75±1.08b	91.20±2.00	1.67±0.04b
75%	3.92±1.16b	89.81±1.34	1.98±0.11ab
90%	2.78±0.53b	90.72±2.45	1.92±0.01ab
100%	7.11±2.27a	89.64±1.94	2.00±0.22ab
Recovered Lipids			
Acid Concentration	Lipid	Protein	Ash
30%	89.45±2.63	13.24±3.811	1.76±0.86a
45%	88.68±3.75	10.66±2.95	1.22±0.26ab
60%	89.50±2.56	10.35±1.07	1.11±0.12ab
75%	91.95±1.84	8.90±3.35	0.94±0.03ab
90%	90.95±1.51	8.38±2.35	0.87±0.17ab
100%	89.97±0.80	8.42±1.56	0.31±0.18b
Recovered Insolubles			
Acid Concentration	Lipid	Protein	Ash
30%	23.57±10.86	59.88±7.05	16.86±4.09
45%	24.61±13.45	53.27±7.78	23.80±1.27
60%	29.36±3.10	54.87±6.33	22.05±4.19
75%	18.94±3.1	57.92±6.23	22.48±5.57
90%	25.86±2.87	62.97±3.85	21.92±4.86
100%	34.26±14.11	57.60±11.78	18.98±7.74

^a Data are given as mean ± standard deviation (n=3). Values in a column with different letters are significantly different (Tukey's honestly significant difference test, $p < 0.05$).

For comparison, proximate analysis of headed gutted silver carp: moisture 75.4% (wet basis), total fat 32.2% (dry basis), crude protein 60.2% (dry basis) and ash 9.2% (dry basis).

Table 2. Reduction of *S. aureus* by ISP processing with 30% formic and lactic acid (1:1 ratio) in silver carp. Values were determined by subtraction of the log of the recovered cells within a fraction from the log of the total initial inoculation (average: 7.10 log CFU/g).. There were significant differences in recovery from **BP** and **TSA** ($P < 0.05$).

Fraction	pH	Log Reduction	
		(mean log CFU/g $\pm$ SD, $n = 3$)	
		BP	TSA
Insoluble	11.5	0.53$\pm$0.56^a	1.67$\pm$0.24^a
	12.5	3.88 $\pm$ 0.45 ^b	3.46 $\pm$ 0.47 ^b
Lipid	11.5	1.36$\pm$0.38^a	2.32$\pm$0.10^a
	12.5	3.54 $\pm$ 0.40 ^b	3.45 $\pm$ 0.27 ^b
Protein	11.5	0.62$\pm$0.15^a	1.65$\pm$0.22^a
	12.5	2.48 $\pm$ 0.77 ^b	2.73 $\pm$ 0.76 ^a
Water	11.5	3.53 $\pm$ 0.67 ^a	4.31 $\pm$ 0.50 ^a
	12.5	5.66 $\pm$ 0.96 ^b	5.41 $\pm$ 0.92 ^a
Total	11.5	0.2 $\pm$ 0.17 ^a	1.29 $\pm$ 0.17 ^a
	12.5	2.41 $\pm$ 0.72 ^b	2.53 $\pm$ 0.64 ^b

^{a, b} Values designated with the same letter within a column are not significantly different ($P > 0.05$) as determined by Tukey's HSD. Log reduction values in bold indicate a significant difference in recovery from **BP** and **TSA** ($P < 0.05$).

Figure 1. Recovered *S. aureus* at pH 11.5 on BP and TSA after ISP processing. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$.

Figure2. Recovered *S. aureus* at pH 12.5 after ISP processing. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$.

Chapter IV

A Comparison of the Bactericidal Effectiveness of Hydrochloric or Acetic Acid on *Staphylococcus aureus* in Silver Carp during Isoelectric Solubilization and Precipitation Protein Recovery Process

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Abstract

An effective method to recover protein, called ISP processing, uses extreme pH shifts to solubilize protein and then recover it by precipitation and centrifugation. The bactericidal effectiveness of this process on *Staphylococcus aureus* has not been tested; therefore, the objective of this study was to compare acids used for the pH shift, hydrochloric and acetic acid, on the reduction of this bacterium during ISP processing. Headed and gutted silver carp were inoculated with *S. aureus*, homogenized and brought to the target pH (2.5, 3.0, 11.5, or 12.5) with the addition of glacial acetic acid, concentrated hydrochloric acid, or sodium hydroxide. Protein was solubilized at 4° C for 10 min and centrifuged to remove lipid and insoluble components (bone, skin, insoluble protein and so on). The solubilized protein was brought to the isoelectric point (pH 5.5) with the addition of NaOH, HCl or acetic acid. Microbial analysis was performed on all the fractions (lipid, protein, insoluble and water) and survivors were enumerated on Baird Parker and Tryptic Soy Agar media. The sums of the surviving cells from each component were compared to the initial inoculum numbers. No significant differences were observed between the selective and growth media ($P > 0.05$) except for in the insoluble fractions. Significant microbial reductions were detected at all acid and pH combinations ($P < 0.05$). The greatest reduction was at solubilization pH 12.5 using acetic acid, with a total reduction of 2.45 CFU/g in the combined fractions and 2.49 CFU/g in the protein fraction. These results show that while ISP will significantly reduce *S. aureus*, further processing is required to achieve a net pasteurization effect.

Introduction

Isoelectric solubilization and precipitation (ISP) processing is a non-thermal method to recover protein and lipid from by-products of fish processing. This is achieved by using extremely acidic or basic pH shifts to solubilize proteins so that insoluble components (skin, bones, scales, etc.) may be separated from them by centrifugation. Proteins precipitate when the solution is returned to the isoelectric point of the protein and then the protein is recovered from the process water by a second centrifugation. Since this is a non-thermal process, it is important to determine the extent at which the process will reduce bacterial load in the recovered protein. When hydrochloric acid (HCl) is used as the processing acid, this process is effective at significantly reducing *Escherichia coli* and *Listeria innocua* in ISP-recovered fish protein (Lansdowne et al., 2009 a & b); however, microbial reductions did not provide a 6-log reduction, which is the reduction amount needed to be considered a pasteurization effect (FDA, 2011).

The pKa defines the strength of an acid on a logarithmic scale and the strength of an acid is defined by the tendency of the acid to give up its proton to water and become dissociated (Gilbert 2000). Strong acids, like HCl, have a low pKa, which means they are mostly in their dissociated form in solutions; however, weak organic acids, like citric and acetic acid, have a higher pKa and are present in both dissociated and undissociated forms in solution. Strong acids dissociate within the solution and outside the bacterial cell. Acid shock or acid stress can occur in low pH conditions when H⁺ ions cross the cell membrane and lower intracellular pH (Abee and Wouters, 1999). On the other hand, organic acids enter the cell in their undissociated form and dissociate once inside the cell. The bactericidal effectiveness of an acid is dependent on the dissociation of the acid within the bacterial cell. The bacterial cell must actively remove the free protons released by the acid and a high concentration of protons will cause cell death by ATP

depletion (Jay et al., 2005). Otto and others (2011b) explored the bactericidal effectiveness of using citric or acetic acids instead of HCl acid as the ISP processing acid. When protein was solubilized under acidic processing conditions, they reported greater reductions in *L. innocua* populations when citric or acetic acid was used as the processing acid when compared to HCl. They also reported a net pasteurization effect (6-log reduction in microbial populations) in all the recovered fractions when acetic acid was used as the ISP processing acid at protein solubilization pH 3.0 (Otto et al., 2011b).

Staphylococcus aureus is a facultative anaerobic Gram-positive coccal bacterium that is mostly found in the nostrils and on the skin and hair of warm-blooded animals (Doyle et al., 2001). Most contaminations of food by *S.aureus* can be traced back to human carriers as well as contaminated equipment that were involved in the preparation process. This bacteria has the ability to tolerate a wide range of temperatures, pH environments, salt concentrations, and is able to survive for extended periods in a dry state (Doyle et al., 2001). Food poisoning by *S.aureus* is a common cause of gastroenteritis which is not due to the ingestion of the live organism but rather is a result of the production of enterotoxin in the staphylococci-contaminated food (Doyle et al., 2001). Symptoms of food poisoning include nausea, violent vomiting, abdominal cramping, sweating, headache, prostration, diarrhea and sometimes a drop in body temperature. Occasional hospitalization is possible for elderly and infants, but the mortality rate is very low (Bore et al., 2007). It is possible that *Staphylococci* exist, at least in low numbers, in all or most animal origin food products or products that have been handled directly by humans and have not been heat processed properly (Bore et al., 2007). Therefore, the objective of this study was to compare the effectiveness of hydrochloric acid to acetic acid on the reduction of *Staphylococcus aureus* during the ISP protein recovery process.

Material and Methods.

Fish Preparation. Fresh silver carp (*Hypophthalmichthys molitrix*) was headed, gutted and sanitized by dipping in a 50 ppm bleach solution for 10 sec (Clorox Regular Bleach, Clorox, Oakland, CA, USA). After draining for an additional 10 sec, fish was placed in a sanitized food processor (BIRO, Marblehead, model 12, Ohio, USA) and ground into a thick paste. Equipment was sanitized by spraying with 70% ethanol and exposed to UV light (254 nm) for 15 min. The paste was divided and 155 g was placed into freezer bags and stored at -80 °C (Lansdowne *et al.*, 2009)

Bacterial strain and inoculum preparation. *Staphylococcus aureus* lab strain (ATCC 25923) was revived in 100 mL sterile brain heart infusion broth in a sterile 250 ml flask (BHI; Becton, Dickinson & Co., Sparks, MD) and incubated at 37°C for 18 to 24 h in a rotary incubator (Barnstead lab-line, model 305, Melrose Park, IL) at 50 rpm. The initial culture was transferred by loop to a second sterile flask containing 100ml brain heart infusion and the same process was repeated. The initial culture was spread onto slants of trypticase soy agar (TSA; Acumedia, Lansing, MI), incubated for 18-24 h at 37°C and stored at 4°C.

S. aureus from working stock slant was twice transferred by loop inocula into 100 mL sterile BHI flasks and incubated at 37°C for 18 to 24 h at 50 rpm in a rotary incubator. Contents were transferred to sterile centrifuge bottles and centrifuged at 10,000 x G for 10 min at 4°C (Otto *et al.*, 2011a & b). Supernatant was removed and pellet was used to inoculate fish paste.

Inoculation of Fish Paste. Fish paste was thawed at 4°C for 18 to 24 h and emptied into an autoclaved ceramic dish. The *S. aureus* pellet was mixed into the paste using an autoclaved spatula. The inoculated paste was immediately used for ISP processing. (Otto *et al.*, 2011 a & b)

Isoelectric solubilization/precipitation. After inoculation, 155 g of fish paste was placed in an autoclaved beaker and homogenized with 930 mL distilled (1:6 wt/vol), deionized water. Homogenizer (BIRO, Marblehead, model 12, Ohio, USA) was sanitized by spraying with 70% ethanol and placing under UV light for 15 min. Glacial acetic acid (Fischer Scientific, Fairlawn, N.L., U.S.A.) , 6N hydrochloric acid or 10N sodium hydroxide was added to the mixture until the target pH (2.5, 3.0, 11.5 or 12.5) was reached. Once pH was adjusted, homogenization was continued for an additional 10 min to allow protein solubilization. The homogenized mixture was poured into autoclaved centrifuge bottles and centrifuged at 10,000 x G for 10 min at 4°C (Otto et al., 2011 a & b). The supernatant (solubilized protein) was poured through autoclaved cheesecloth to collect the lipid. Lipid and insoluble portions remaining on the bottom of the centrifuge tubes were retained in sterile containers for microbial analysis. The supernatant was brought to its isoelectric point (pH 5.5) by the addition of 10N NaOH, glacial acetic acid or 6N HCl and homogenized for an additional 5 min. The solution was poured into autoclaved centrifuge bottles and was centrifuged at 10,000 x G for 10 min at 4°C (Otto et al., 2011 a & b). Both supernatant and precipitated proteins were retained separately in sterile containers for microbial analysis.

Microbial Analysis. Microbial load was determined on uninoculated fish paste (background), inoculated fish paste (initial), and recovered fractions (protein, lipid, insoluble and water). An aliquot (1 g) was placed into separate stomacher bags with 9 ml sterile peptone buffer and hand-pummeled for 2 min. Pummeled samples were serially diluted (10-fold) and spread-plated onto TSA (non-selective) and Baird Parker medium (BP; selective) plates and incubated at 37°C for 24 h. The detection limit for non-inoculated fish paste was $<10^3$ CFU/g, for lipid, protein and insoluble fractions it was $<10^2$ CFU/g and for water it was <10 CFU/g. (Otto et al., 2011 a & b) Total log reduction was calculated by subtracting the summation of survivors in all fraction

from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$ (Lansdowne et al., 2009 a & b).

Statistical Analysis. This study was replicated three times for each acid type and each solubilization pH value. Microbial counts (CFU/g) were converted into logarithmic units and recovered cells were expressed as $\log_{10}$ CFU/g. Data were analyzed by analysis of variance and differences in means were determined using Turkey's honestly significant differences test ($P < 0.05$) (JMP 10, SAS Institute, Cary, NC).

Results and Discussion

For all trials, the mean background flora of the initial fish pasted was 3.4×10^3 CFU/g on TSA and there was no growth of *Staphylococcus aureus* detected ($< 10^2$ CFU/g) on BP. There were no differences ($P > 0.05$) in cell recovery on TSA and BP for the protein, lipids, and water fractions, implying that microbial death due to ISP processing was an “all-or-nothing event” (Table 1). This is consistent with Lansdowne and others (2009 a) and Otto and others (2011 a & b) who found that when *Listeria monocytogenes* and *L. innocua* were exposed to the pH-shift process, there were no differences in recovery on selective or non-selective media. In contrast, when *Escherichia coli* were exposed to HCl using the pH-shift process, there were differences in recovery on selective and non-selective media, implying that cells were injured during the process (Lansdowne et al., 2009b). Bacterial cell injury occurs as a result of exposure to a chemical or physical process that damages but does not kill a microorganism (Hurst et al., 1997). As a result of metabolic and structural injury, microorganisms lose their characteristic growth capabilities and they are unable to form visible colonies under selective conditions, where uninjured cells are

able to form colonies. Most injured cells suffer from structural damage, which could affect the cell wall or membrane permeability; rather than metabolic injury, where the functional components of the cell are damaged (Brashears et al., 2001). In Gram-negative bacteria, i.e. *E. coli*, damage to the outer membrane of the cell causes the release of lipopolysaccharides, lipids, phospholipids, divalent cations necessary for lipopolysaccharide stability, and periplasmic enzymes which may disrupt membrane permeability (Wesche et al., 2009). However, in the study by Lansdowne and others (2009b), the bacteria were in a rich environment provided by fish proteins and other components which may have led to the injury of cells instead of death under basic conditions. Protein rich environments contain carnitines, betaines, and peptides that protect these bacteria from adverse conditions. These components prolong the internal homeostasis of the bacteria and allow it to survive in acidic or basic conditions (Russell et al., 1995).

The greatest microbial reductions ($P < 0.05$) occurred when protein was solubilized at pH 12.5 for both processing acids, with a total log reduction of 2.45 CFU/g and 1.86 CFU/g for acetic and HCl acids, respectively (Table 2). These data are consistent with the results of Ronaghi (2013) who observed that when formic and lactic acid was used as the processing acid during the ISP process, the most bactericidal protein solubilization pH was at pH 12.5. The protein fraction contained the most recovered cells (Figure 1) except at pH 11.5 where most cells were recovered in the insoluble fraction and water contained the fewest. The high numbers of recovered cells in the insoluble fractions is most likely due to centrifugation which would leave fewer cells left within the protein and water fractions (Lansdowne et al., 2009a). According to the U.S. Food and Drug Administration, pasteurization is defined as a 6-log reduction in microbial populations. A net pasteurization effect was not observed at any of the pH conditions. When microorganisms are placed in an environment below or above neutrality, they need to bring the environmental pH to a

more optimum value to survive and proliferate (Burl et al., 1999). Aspects of cell function such as nutrient acquisitions, energy generation, pH homeostasis and protection of components may be affected by acidic conditions (Rode et al., 2010). In acidic conditions, bacterial cells must either keep protons from entering or expel them as fast as they enter (Jay et al., 2005). On the other hand, high pH levels may solubilize the bacteria's membrane protein and lipids which can lead to the exposure of hydrophobic sites of adjacent lipids to the environment (Jacobsohn et al., 1992). In Gram-negative bacteria, damage to the membrane protein and lipids by high pH as well as the exposure of hydrophobic areas of adjacent phospholipids can lead to the disruption of the stability of the membrane and predispose it to rupture by intracellular turgor pressure (Mendonca et al., 1994). However, Gram-positive bacteria such as *L. monocytogenes* are able to maintain their overall integrity when exposed to high pH conditions due to the presence of the peptidoglycan cell wall (Shockman and Barrett 1983). The cell wall is able to prevent the swelling and ultimate bursting of the cell by stabilizing the membrane against the turgor pressure exerted by the cytoplasm (Csonka, 1989). Bacteria cells may also use cytoplasmic buffering by synthesizing intracellular metabolites in an attempt to stabilize pH (Dilworth and Glenn 1999). These mechanisms might explain the generally low number of log reduction in *S. aureus* populations, but they are only a temporary fix to oppose the increasing OH^- concentration.

Strong acids such as HCl completely dissociate in solutions. The protons tend to pass through the membrane by interacting with the systems that control the proton flow in and out of the cell, such as the electron transport system. On the other hand, weak organic acids exist in a pH-dependent equilibrium between the dissociated and undissociated state in solutions. The dissociation of an acid depends on temperature, pH and the dissociation constant of the acid (Rode et al., 2010). These acids have optimal antimicrobial activity at low pH levels because they

are mostly in their uncharged and undissociated form which easily permeates through the membrane of bacterial cells. Once inside the cell, the molecules will dissociate and release charged anions and protons which will not cross the cellular membrane. The accumulation of these protons and anions will eventually cause membrane disruption (Burl et al., 1999). In the present study, HCl acid was shown to be more effective ($P < 0.05$) at reducing *S. aureus* than acetic acid when protein was solubilized under acidic processing conditions (Table 2); however, it is important to point out that microbial reductions were very low, with the average total reduction being less than 1 log. Perhaps increasing the exposure time of *S. aureus* to these acids could result in higher log reductions and would allow us to differentiate between the effectiveness of acids used during ISP.

Otto and others (2011b) determined the effect of organic acids (citric and acetic) on the reduction of *L. innocua* during ISP processing of rainbow trout. Significant reductions ($P < 0.05$) were observed in microbial reductions regardless of pH or acid type. A 6.41 log CFU/g reduction was seen in the recovered protein fraction at processing pH 3.0 with acetic acid, compared to a 1.11 log CFU/g reduction when HCl was used as the processing acid (Lansdowne et al., 2009). However, when the same organic acids and processing pHs were utilized during ISP processing of rainbow trout inoculated with *L. monocytogenes*, only a 3.53 CFU/g reduction occurred in the protein fraction (Otto et al., 2011a).

In another study, Lansdowne and others (2009) determined the effect of ISP processing at acidic (2.0 and 3.0) and basic (11.5 and 12.5) pH conditions on the survival of *L. innocua*. They did not observe a net pasteurization effect while using HCl during ISP processing at any of the processing pHs. The greatest reduction of *L. innocua* in the protein fraction was observed at processing pH 2.0 which resulted in a 3.8 log reduction and the least reduction occurred at pH

11.5 (Lansdowne et al., 2009). In contrast, the results of this current study that solubilizing protein at pH 2.5 resulted in a total log reduction of less than one in *S. aureus* populations; the greatest reduction were observed when protein was solubilized at pH 11.5 and 12.5.

Conclusion

The current study indicates that ISP is not an effective process in eliminating *Staphylococcus aureus* populations. Although, the collected data suggests that weak organic acids are as effective as strong inorganic acids when used during the ISP process at reducing *S. aureus*; however, the low reductions in microbial populations do not allow us to make a definite conclusion. Basic pH conditions seem to have a better effect on the reduction of *S. aureus* during ISP processing. To further investigate the effect of ISP on *S. aureus*, future research should focus on increasing the exposure time of *S. aureus* to high or low pH conditions and possibly increasing the initial inoculation levels to better observe the effect of different acids.

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Table 1. Log reduction of *S. aureus* by ISP processing with acetic and hydrochloric acids in silver carp. Values were determined by subtraction of the log of recovered cells within a fraction from the log of the total initial inoculation (average: 7.10 log CFU/g).

Fraction	pH	Acid	Log Reduction (mean log CFU/g $\pm$ SD, $n=3$)		Fraction	pH	Acid	Log Reduction (mean log CFU/g $\pm$ SD, $n=3$)	
			BP	TSA				BP	TSA
Protein	2.5	Acetic	0.58 $\pm$ 0.17 ^{bc}	0.73 $\pm$ 0.23 ^c	Lipid	2.5	Acetic	1.05 $\pm$ 0.14 ^b	1.06 $\pm$ 0.21 ^b
		HCl	0.97 $\pm$ 0.12 ^{bc}	1.13 $\pm$ 0.19 ^c			HCl	1.80 $\pm$ 0.13 ^b	2.00 $\pm$ 0.29 ^b
	3.0	Acetic	0.31 $\pm$ 0.27 ^c	0.36 $\pm$ 0.32 ^c		3.0	Acetic	1.09 $\pm$ 0.17 ^b	1.26 $\pm$ 0.36 ^b
		HCl	0.93 $\pm$ 0.29 ^{bc}	0.90 $\pm$ 0.33 ^c			HCl	1.83 $\pm$ 0.14 ^b	1.79 $\pm$ 0.11 ^b
	11.5	Acetic	1.03 $\pm$ 0.39 ^{bc}	1.23 $\pm$ 0.21 ^c		11.5	Acetic	1.35 $\pm$ 0.60 ^b	1.60 $\pm$ 0.44 ^b
		HCl	1.04 $\pm$ 0.14 ^{bc}	1.42 $\pm$ 0.41 ^{bc}			HCl	0.77 $\pm$ 0.44 ^b	1.46 $\pm$ 0.85 ^b
	12.5	Acetic	2.22 $\pm$ 0.54 ^a	2.77 $\pm$ 0.60 ^a		12.5	Acetic	4.17 $\pm$ 0.60 ^a	4.25 $\pm$ 0.78 ^a
		HCl	1.46 $\pm$ 0.35 ^{ab}	2.34 $\pm$ 0.60 ^{ab}			HCl	3.64 $\pm$ 0.82 ^a	3.96 $\pm$ 0.68 ^a
Insoluble	2.5	Acetic	0.99 $\pm$ 0.21 ^{bc}	0.99 $\pm$ 0.09 ^{bc}	Water	2.5	Acetic	4.10 $\pm$ 0.51 ^a	4.15 $\pm$ 0.08 ^a
		HCl	1.64 $\pm$ 0.06 ^b	2.02 $\pm$ 0.37 ^b			HCl	4.39 $\pm$ 0.36 ^a	4.05 $\pm$ 0.89 ^a
	3.0	Acetic	1.13 $\pm$ 0.05 ^{bc}	1.25 $\pm$ 0.22 ^{bc}		3.0	Acetic	4.09 $\pm$ 0.97 ^a	4.16 $\pm$ 1.03 ^a
		HCl	1.37 $\pm$ 0.20 ^{bc}	1.32 $\pm$ 0.15 ^{bc}			HCl	4.38 $\pm$ 0.39 ^a	4.02 $\pm$ 0.35 ^a
	11.5	Acetic	0.70 $\pm$ 0.21^{cd}	1.20 $\pm$ 0.07^{bc}		11.5	Acetic	4.03 $\pm$ 0.61 ^a	3.93 $\pm$ 0.35 ^a
		HCl	0.10 $\pm$ 0.17 ^d	0.58 $\pm$ 0.34 ^c			HCl	4.16 $\pm$ 0.20 ^a	4.52 $\pm$ 0.23 ^a
	12.5	Acetic	3.46 $\pm$ 0.62 ^a	4.42 $\pm$ 1.08 ^a		12.5	Acetic	5.15 $\pm$ 0.77 ^a	5.63 $\pm$ 1.16 ^a
		HCl	2.72 $\pm$ 0.35^a	4.07 $\pm$ 0.49^a			Acetic	4.68 $\pm$ 0.16 ^a	5.27 $\pm$ 0.40 ^a
Total	2.5	Acetic	0.26 $\pm$ 0.24 ^c	0.36 $\pm$ 0.10 ^b					
		HCl	0.82 $\pm$ 0.11 ^{bc}	1.02 $\pm$ 0.20 ^b					
	3.0	Acetic	0.20 $\pm$ 0.17 ^c	0.26 $\pm$ 0.24 ^b					
		HCl	0.74 $\pm$ 0.23 ^{bc}	0.70 $\pm$ 0.27 ^b					
	11.5	Acetic	0.33 $\pm$ 0.35 ^c	0.79 $\pm$ 0.20 ^b					
		HCl	0 ^c	0.28 $\pm$ 0.49 ^b					
	12.5	Acetic	2.18 $\pm$ 0.53 ^a	2.73 $\pm$ 0.61 ^a					
		HCl	1.42 $\pm$ 0.35 ^{ab}	2.30 $\pm$ 0.56 ^a					

^{a, b, c} Values designated with different letters within a column and fraction are significantly different ($P < 0.05$) as determined by Tukey's HSD. Values in **bold** indicate a significant difference in recovery between **BP** and **TSA** ($P < 0.05$).

Table 2. Log reduction of *S. aureus* exposed to several pH shifts in different fractions of headed and gutted silver carp. Values were determined by subtraction of the log of recovered cells within a fraction from the log of the total initial inoculation (7.10 log CFU/g). Combined data from TSA and BP There were no significant differences in recovery between TSA and BP ($P > 0.05$).

Fraction	pH	Acid	Log Reduction (mean log CFU/g $\pm$ SD, n=6)
Protein	2.5*	Acetic	0.68 ± 0.20 ^{cd}
		HCl	1.05 ± 0.16 ^{cd}
	3.0*	Acetic	0.34 ± 0.26 ^d
		HCl	0.92 ± 0.28 ^{cd}
	11.5	Acetic	1.13 ± 0.30 ^c
		HCl	1.23 ± 0.34 ^{bc}
	12.5	Acetic	2.49 ± 0.59 ^a
		HCl	1.90 ± 0.66 ^{ab}
Lipid	2.5*	Acetic	1.06 ± 0.16 ^b
		HCl	1.90 ± 0.23 ^b
	3.0*	Acetic	1.17 ± 0.27 ^b
		HCl	1.81 ± 0.15 ^b
	11.5	Acetic	1.47 ± 0.49 ^b
		HCl	1.12 ± 0.71 ^b
	12.5	Acetic	4.21 ± 0.62 ^a
		HCl	3.80 ± 0.70 ^a
Total	2.5*	Acetic	0.31 ± 0.17 ^{bc}
		HCl	0.92 ± 0.18 ^b
	3.0*	Acetic	0.23 ± 0.19 ^{bc}
		HCl	0.72 ± 0.23 ^{bc}
	11.5	Acetic	0.56 ± 0.36 ^{bc}
		HCl	0.17 ± 0.38 ^c
	12.5	Acetic	2.45 ± 0.59 ^a
		HCl	1.86 ± 0.64 ^a

* Indicates significant difference in reductions between acids within treatment ($P < 0.05$)

a, b, c, d Values designated with the different letters within a column and fraction are significantly different ($P < 0.05$)

Figure 1. Recovered *S.aureus* after ISP processing of silver carp at acidic processing pH levels. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$.

Figure 2. Recovered *S.aureus* after ISP processing of silver carp at basic processing pH levels. The left pie chart indicates the percentage of recovered cells from the initial inoculum and the right pie chart indicates the percentage of recovered cells in each fraction. Total log reduction was calculated by subtracting the summation of survivors in all fraction from the initial inoculum: $\log(\text{initial inoculum}) - \log(\text{survivors in protein fraction} + \text{insoluble fraction} + \text{lipid fraction} + \text{water fraction})$. The log reduction in each fraction: $\log(\text{initial inoculum}) - \log(\text{survivors in specific fraction})$.