Investigating the ability of spice extracts to inhibit bacterial growth and/or histamine accumulation associated with scombroid food poisoning

by

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ABSTRACT

Feseekh, a fermented fish dish, is eaten the Monday following Easter. When feseekh fermentation produces unsafe histamine levels, consumption causes scombroid food poisoning. Many bacteria have been implicated, including *Morganella morganii*, the bacterium used in this study. This study's purpose was to determine whether spices normally used in this fermented dish influence bacterial growth and histamine accumulation. Spice extracts were used in a disk diffusion assay with results showing no spice-induced growth inhibition. Next, histamine levels were measured in laboratory medium and in fish fermentations. No statistical differences in histamine were observed. Closer examination of the data revealed complicating issues. Extracts showed high background, suggesting they may contain confounding enzymes. Additionally, the low histamine levels, in combination with the high variability in the assay, may have disguised inhibition effects. Future directions include the use of direct assays to determine histamine levels and the use of fresh fish in fermentations.

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Introduction

In Poland, the coming of spring is celebrated by throwing handmade Marzannas, or straw dolls, into the water and drowning it to say goodbye to the wrath of winter (Galimberti, "10 unique spring traditions from around the world"). In Switzerland, a celebratory show consisting of a burning snowman takes place to welcome spring. In Japan, people await the cherry blossoms, which they then look at by taking a boat out into the water. In Egypt, spring is welcomed by eating a traditional fish which is made specifically for this day, called feseekh (Figure 1). It is always celebrated on the Monday following the Coptic Orthodox Easter ("Sham El-Nessim 2019"). On Sham El-Nessim, or the day on which this is celebrated, people gather at the park with all of their family and friends and they have a picnic consisting of this salted fish and spring onions.

Feseekh is a fermented mullet fish that is prepared with salt and different spices and is usually kept in warm environments (Berger 2017). Depending on how it is made and how long it is kept, feseekh can cause scombroid poisoning, which can lead to death. Scombroid poisoning occurs when an individual has eaten fish with very high levels of histamine (Stratta and Badino 2012).

Figure 1. Mullet fish before it was prepared to be used for this experiment. This is the mullet fish that was used for this experiment before it was fermented. To prepare it, it was descaled and degutted and was then filleted to be used.

High levels of histamine can cause headaches, hives, fevers, and heart palpitations (Stratta and Badino 2012). Histamine levels depend on how the fish was prepared and/or stored. High levels of histamine in fish are not apparent in either the color or the odor, so it cannot be determined if the fish is safe to eat by looking at it or smelling it. Even though fish is known to contain essential amino acids, those free amino acids do change once the fish is fermented for various periods of time, such as when making feseekh (Mohamed *et al*. 2009).

Feseekh fermentation is a natural salt fermentation in that the fish is not inoculated with specific microorganisms, but those already naturally present in the air, water, and fish ferment the dish. Many different bacteria have been implicated in the feseekh fermentation process, including *Morganella morganii, Morganella psychrotolerans, Klebsiella pneumoniae, Hafnia alvei, Photobacterium phosphoreum, Photobacterium psychrotolerans, Acinetobacter lowff, Plesiomonas shigelloides, Pseudomonas putida, Pseudomonas fluorescens* and *Aeromonas spp* (Biji *et al*. 2016). This study will use *Morganella morganii* as a representative feseekh fermentation bacterium because it produces high levels of histamines under experimental conditions at about 2765 mg/kg of histamine (López-Sabater *et al*. 1994). In other feseekh studies, histamine was found at levels as high as 521 mg/kg (Rabie *et al*. 2011). *M. morganii* is a gram-negative bacterium found in the gut and intestinal tract of fish, has been found in feseekh preparations, and is known to have decarboxylase enzymes which convert amino acids in the fish into biogenic amines, such as histamine.

Histamine is one of the most common biogenic amines found in various foods and it is also one that is significantly studied. Ingestion of histamines is the cause of a distinct

type of food poisoning called scombroid poisoning. The range of 8-40 mg of histamine consumption causes a slight reaction to the person who ingested it, such as sweating or mild drowsiness (Biji *et al*. 2016). The range of 40-100 mg is considered an intermediate stage, resulting in some of the milder symptoms that include headaches and nausea. Any consumption over 100 mg may cause very severe symptoms, such as diarrhea, difficulty breathing, and could even be as severe as death, at very high levels. The histamine levels in the fish are dependent on how long the fish was fermented (ripened) and stored. Feseekh is considered safe to eat when it is consumed 20-40 days after it has been fermented. It is considered dangerous 60 days after fermentation because of the high histamine levels.

Spices are also used consistently in feseekh preparation. Traditionally, spices have been used throughout history to add flavoring to food, treat different diseases, reduce bacterial and fungal growth, and preserve food (Liu *et al.* 2017). Clove, for example, contains antiseptic properties that are present in medicine to help treat periodontal disease because of its antimicrobial properties against oral bacteria. The same properties can be seen in oregano and thyme. When used in a disk diffusion assay, clove had the ability to inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. Even though most feseekh recipes are very similar, most of them incorporate different spices to decrease the odor and give it more flavor, anecdotally. Informally, most feseekh recipes only call for spices such as cumin, paprika, and turmeric along with salt (Appendix).

Traditional feseekh spices contain compounds that allow them to act as antiseptics. Cumin (*Cuminum cyminum*) contains the compound cuminal, paprika (*Capsicum annuum*) contains capsaicin, and turmeric (*Curcuma longa*) contains

curcumin, all of which help give these spices their antimicrobial effects (Gottardi *et al.* 2016). Cuminal, which is also known as cuminaldehyde, 4-isopropylbenzyaldehyde or 4-propan-2-ylbenzaldehyde, is a water insoluble terpenoid that is categorized as a flavoring agent with an acid, green, herb, sharp flavor profile from the Flavor and Extract Manufacturers Association (PubChem CID 326). Cumin-derived compounds have been shown to inhibit aldose reductase and alpha-glucosidase (Lee 2005). Volatile cumin oil and cuminaldehyde are more effective as antimicrobial agents against fungi (*Saccharomyces, Candida, Aspergillus, Penicillium*) than bacterial species *(Escherichia, Pseudomonas, Staphylococcus*) (Shetty *et al.* 1994). Capsaicin, which is also known as (*E*)-*N*-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide, is a water soluble capsacinoid that is categorized as a flavoring by the Center for Food Safety and Applied Nutrition (PubChem CID 1548943). It is a known agonist for trans-membrane receptor-ion channel complex (TRVP) and has been shown to influence mitochondrial respiration and pain sensation (Drug Bank ID DB06774). Its ability to inhibit pain sensation (nociception) has led to its use as an analgesic. Capsaicinoids are known to be effective antimicrobial agents against the plant fungal pathogen species *Fusarium* (Tewksbury *et al*. 2008). Lastly, curcumin, which is also known as (1*E*,6*E*)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione, is a phytopolyphenol pigment that is categorized as a flavoring and color additive by the Joint FAO/WHO Expert Committee on Food Additives (PubChem CID 969516). It is a known inhibitor of cyclooxygenases, which makes it effective at reducing reactive oxygen species. Curcumin's antimicrobial properties have been shown to be due to membrane disrupting functions (Tyagi *et al.* 2015).

Historically, use of these spices have been implicated in making the dish safer to consume, however, no empirical evidence has been gathered to refute or support this idea. To test this, the ability of these three spices as dry solids and as compounds extracted by oil and water were used to determine if each had the ability to affect *M. morganii* growth and histamine production. The hypothesis for this research project was that at least one spice or spice extract would inhibit the growth and/or the histamine production by *Morganella morganii*, which is a representative feseekh bacterium. If this hypothesis can be proven, then this research project shows specific spices might be used to make and prepare this fish safely, at least in relation to *M. morganii.*

Materials and Methods

Preparation of Spice Extracts

The three spices used in this experiment were paprika (Publix brand), cumin (Publix brand), and turmeric (Spice Islands brand). For each spice, three different replicate extracts were made using water (sterile reverse osmosis) and canola oil (Publix brand). First, 0.5 g of each spice was combined with 5 mL of sterile water or 5 mL of canola oil in a sterile 50 mL conical tube. Each tube was mixed thoroughly and then incubated at room temperature with shaking at 45 RPM for one week, to ensure the spice was constantly mixing with the oil or water and releasing as much of its antibacterial properties. Each tube was centrifuged at 14,000 rpm for 5 minutes and the liquid extract was removed to a fresh tube. The tubes with paprika and water were centrifuged at 14,000 rpm 2-3 more times than the other samples to generate extracts without fibrous debris.

Preparation of Bacterial Cultures

To prepare the *Morganella morganii* cultures for all experiments, Luria Broth (LB) (Difco™ LB Broth Lennox, BD) and Tryptic Soy Broth (TSB) (Sigma Aldrich Fine Chemicals Biosciences) were both used for the same purposes. Each culture was made by inoculating a single colony of *M. morganii* (ATCC# 25829) into either 2 mL of LB or TSB. The culture was then grown at 37°C for at least 4 hours.

Disk Diffusion Assay

To prepare the bacterial plates, a sample of *Morganella morganii* was inoculated into 2 mL LB and grown at 37°C overnight. Mueller Hinton agar (Difco™ Mueller Hinton Agar) plates were inoculated in a lawn using 50 µL of bacteria into a pool of sterile water and was used immediately.

To prepare disks permeated with extracts, sterile 6 mm paper disks (Becton, Dickinson and Company BBL™) were placed onto a sterile dish and 30 µL of extract, kanamycin (250 mg/mL) (Kanamycin Sulfate, Fisher BioReagents), canola oil, or sterile water was used to saturate the disk. All the disks were placed at 37°C for one hour to dry before placing on the Mueller-Hinton agar plates inoculated with *M. morganii.* All the plates were incubated at 37°C overnight. At 18 hours, zones of inhibition were noted.

Testing Histamine Levels of M. morganii in Laboratory Medium

The histamine assay kit (EnzyChrom™ Histamine Assay Kit; EHIS-100) (Figure 2) was used following the manufacturer's directions to determine whether *M. morganii* produced detectable levels of histamine in the laboratory medium, either in TSB or LB. To prepare the culture, a sample of *M. morganii* was inoculated into 2 mL LB and grown at 37°C for overnight. Cells were pelleted by centrifugation and the supernatant was used directly and diluted for the assay. The concentrations tested in the assay were 100%, 20%, and 5%. The 100% dilution contained only *M. morganii*, the 20% dilution contained 1:5 ratio of bacteria to TSB, and the 5% dilution contained a 1:20 ratio of bacteria to TSB. A standard curve was prepared as directed by the manufacturer's directions (data not shown).

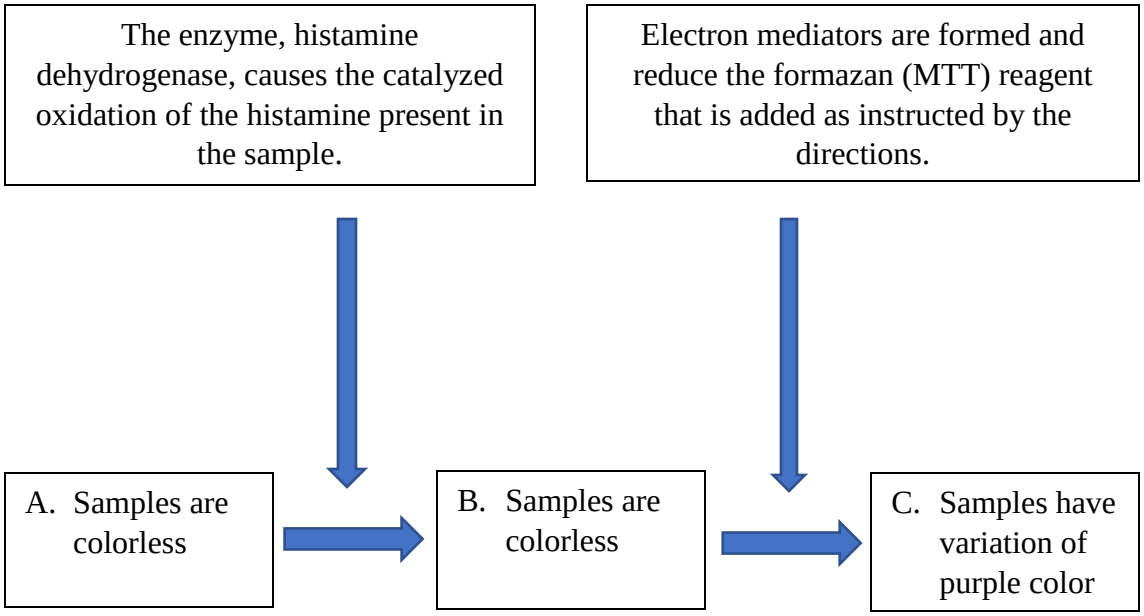


Figure 2. Intensity of product color is correlated to histamine levels present in samples. This shows the steps of how the enzyme, histamine dehydrogenase, catalyzes the reaction and produces the purple color, as well as how color intensity is proportionally corelated to histamine levels in the samples.

Testing Histamine Levels in Fish Samples with and Without Bacterial Inoculation

Morganella morganii was prepared by inoculating 2 mL LB and grown at 37°C for 4 hours. Previously frozen salmon was purchased and used in this experiment. One hundred mg of fish, 100 mg of spice, and 100 µL canola oil was incubated at room temperature for 3 days with 1 µL sterile water or bacterial culture. The tissue was then minced and extracted with 0.5% acetic acid as directed by the manufacturer of the histamine assay kit for determining histamine levels from tissues. A standard curve was prepared as directed by the manufacturer's directions (data not shown).

Testing Histamine Levels in Fish Samples with Bacterial Inoculation

M. morganii was prepared by inoculating 2 mL TSB and growing at 37°C for 5 hours. Previously frozen mullet fish was used in a second experiment to test histamine levels. This experiment followed the same measurements of fish, spice, and canola oil as the previous one. The samples were incubated at room temperature for 3 weeks with 1 µL bacterial culture. The tissue of the fish was then minced and extracted using 0.5% acetic acid as directed by the manufacturer's directions of the histamine assay kit to determine the histamine levels in the fish. A standard curve was prepared as directed by the manufacturer's directions (data not shown).

Results

Spices have been known to reduce food spoilage (Liu *et al.* 2017) and are included in many fermented foods. The objective of this study was to determine whether cumin, paprika, and turmeric, which are used in most preparations of a fermented dish, had any effect on accumulation of histamine. First, it was important to establish whether any of the three spices had antibacterial properties and could thus affect histamine levels by simply inhibiting bacterial growth. To test this, spices were extracted with oil and water in triplicate (Figure 2) and used in a disk diffusion assay with a representative bacterium, *Morganella morganii*. However, none of the spice extracts showed any zones of inhibition and were identical to the negative control (sterile water) (Figure 3-5), while zones of inhibition were observed with the positive control, kanamycin.

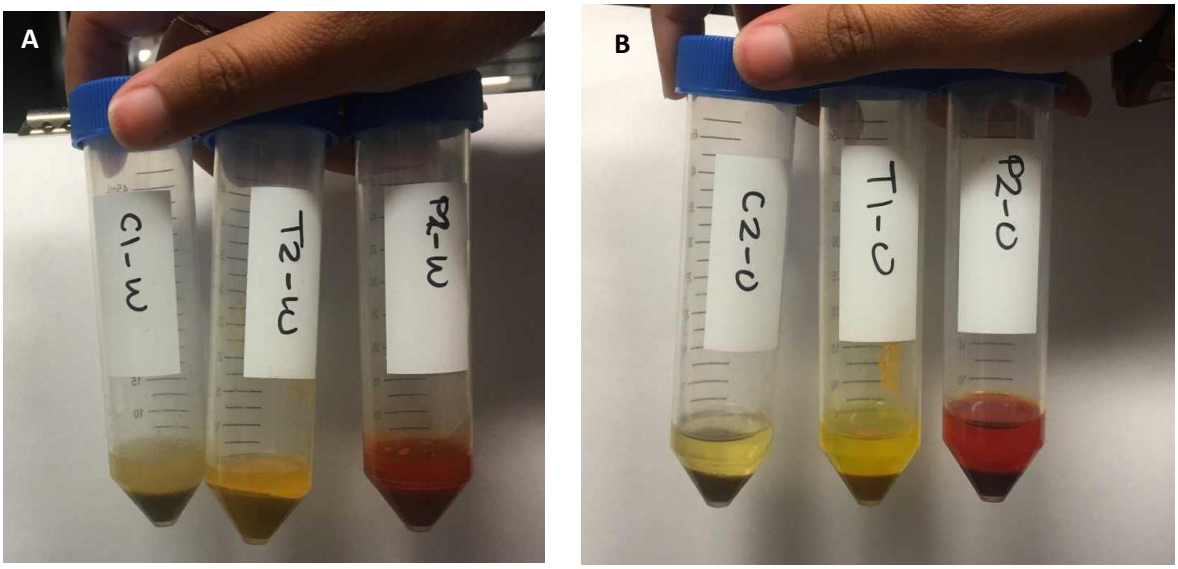
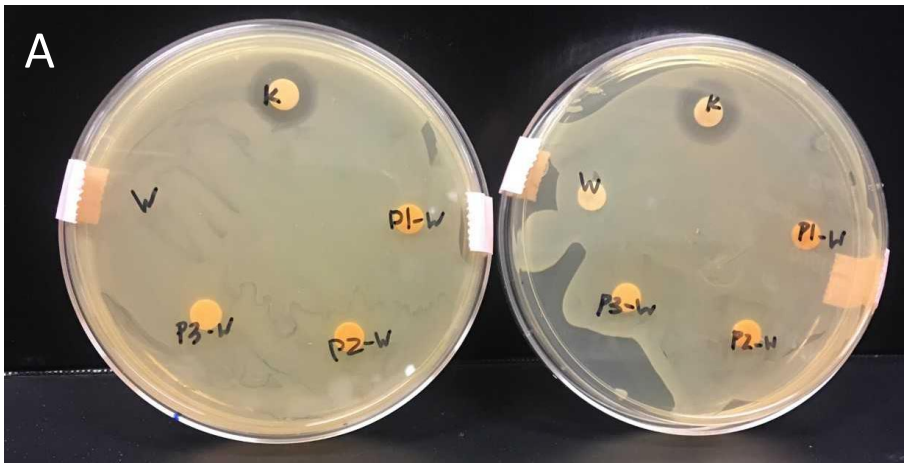


Figure 3. Paprika, cumin, and turmeric water and oil extract preparation. A. Spice-water and B. spice-oil extracts after centrifugation showing the liquid and fibrous layers. Labels indicate the following: C1-W: Cumin + water, replicate 1; T2-W: turmeric + water, replicate 2; P2-W: paprika + water, replicate 2; C2-O: cumin + oil, replicate 2; T1-O: turmeric + oil, replicate 1; P2-O: paprika + oil, replicate 2.

Paprika

Water



Oil

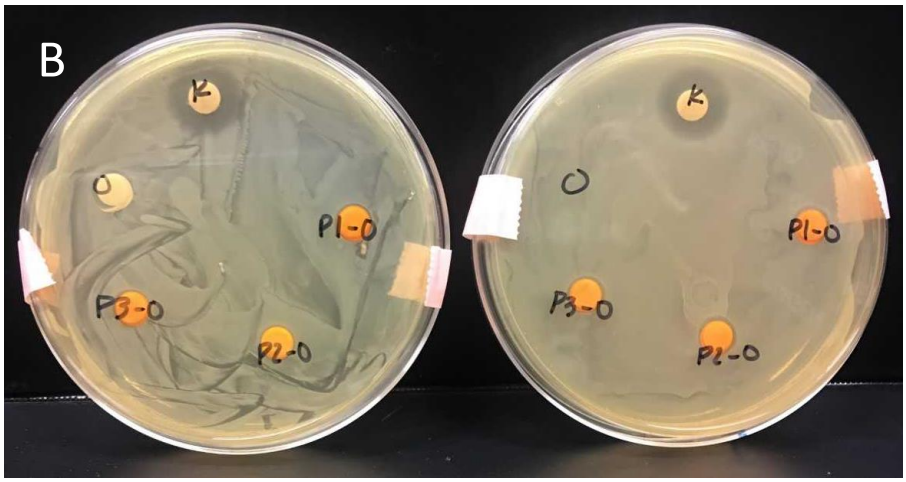


Figure 4. Disk diffusion results show no spice-related growth inhibition for paprika. Labels indicate the following: A, B. Water or oil-extracted paprika-induced zone of inhibition. K: Kanamycin. O: Oil. W: Water. P1W: Disk contains paprika water extract, 1st replicate. P2W: Disk contains paprika water extract, 2nd replicate. P3W: Disk contains paprika water extract, 3rd replicate. P1O: Disk contains paprika oil extract, 1st replicate. P2O: Disk contains paprika oil extract, 2nd replicate. P3O: Disk contains paprika oil extract, 3rd replicate.

Cumin

Water

Oil

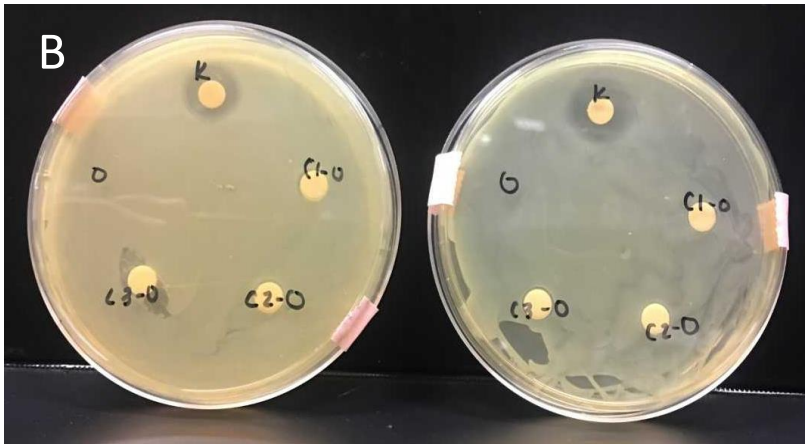


Figure 5. Disk diffusion results show no spice-related growth inhibition for cumin. Labels indicate the following: A, B. Water or oil- extracted cumin-induced zone of inhibition. K: Kanamycin. O: Oil. W: Water. C1W: Disk contains cumin water extract, 1st replicate. C2W: Disk contains cumin water extract, 2nd replicate. C3W: Disk contains cumin water extract, 3rd replicate. C1O: Disk contains cumin oil extract, 1st replicate. C2O: Disk contains cumin oil extract, 2nd replicate. C3O: Disk contains cumin oil extract, 3rd replicate.

Turmeric

Water

Oil

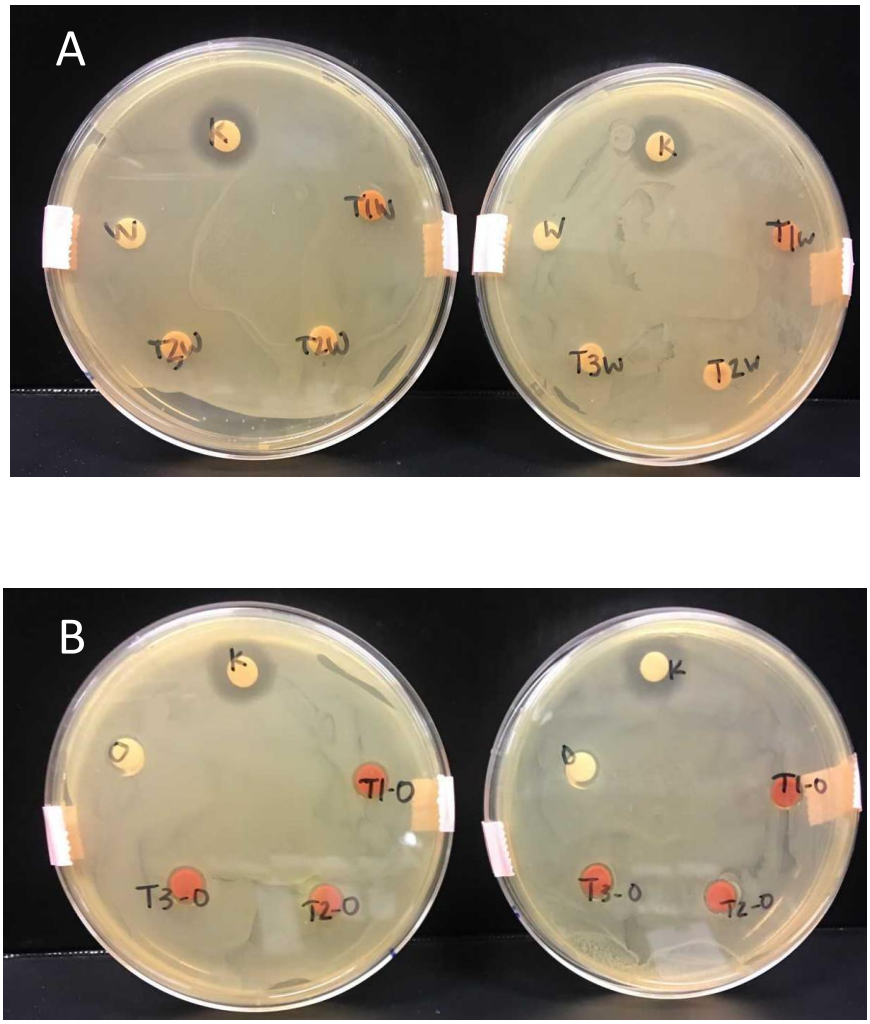


Figure 6. Disk diffusion results show no spice-related growth inhibition for turmeric. Labels indicate the following: A, B. Water or oil- extracted turmeric-induced zone of inhibition. K: Kanamycin. O: Oil. W: Water. T1W: Disk contains turmeric water extract, 1st replicate. T2W: Disk contains turmeric water extract, 2nd replicate. T3W: Disk contains turmeric water extract, 3rd replicate. T1O: Disk contains turmeric oil extract, 1st replicate. T2O: Disk contains turmeric oil extract, 2nd replicate. T3O: Disk contains turmeric oil extract, 3rd replicate.

Since none of the traditional feseekh spices inhibited the growth of *M. morganii*, histamine production by the representative bacteria in lab medium was investigated next, as this would allow complete control of growth conditions. To determine whether *M. morganii* would produce sufficient histamine levels for an inhibition type experiment, a histamine assay was used to determine histamine levels in liquid broth that had been inoculated with *M. morganii* and grown overnight. The source of histidine came from the tryptic soy broth that *M. morganii* was grown in. The levels of histamine were detectible, but low (Figure 6). With such low levels of histamine in the lab culture, it would make it very difficult to see any inhibition caused by the spices.

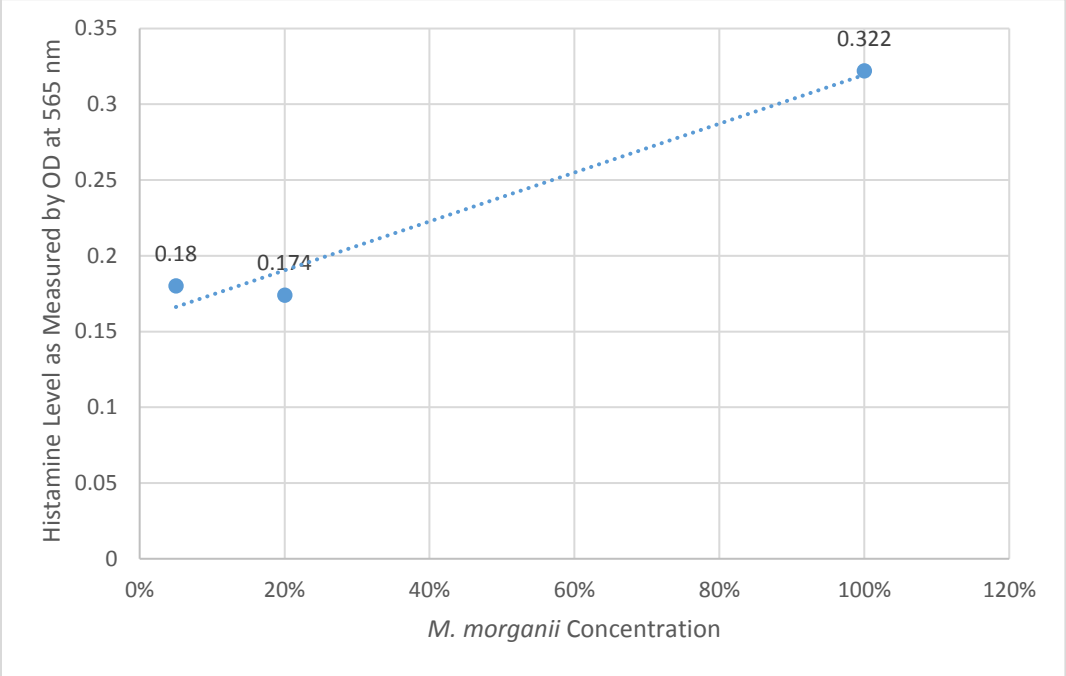


Figure 7. *M. morganii* produces low histamine levels in laboratory growth medium. The absorbance values, including at 100% culture concentration, were too small to continue to test for the inhibition of histamine produced by *M. morganii*.

With the *M. morganii* lab culture producing such small amounts of histamine, the next step was to use fish fermentation to induce histamine accumulation. First, it was necessary to determine whether incubation of bacteria already present in fish alone could produce high histamine levels in the laboratory, or whether inoculation of *M. morganii* would be necessary. Histamine levels were tested in previously-frozen salmon that was incubated with or without *M. morganii* for three days (Table 1-4, Figure 7-8). Each sample that was run had three true, independent replicates. To determine whether the values were significantly different from each other, ANOVA was used to compare all the sample absorbances. There was no statistical difference between any of the sample absorbances, as determined by ANOVA. This provides evidence to show how cumin, paprika, and turmeric have no effect on the inhibition of histamine production or histamine accumulation by *M. morganii* in previously frozen salmon.

Table 1. Absorbances taken at 565 nm in salmon natively fermented for three days with no added *M. morganii* and with the assay enzyme, histamine dehydrogenase.

The absorbance values produced by the salmon experiment contained enough variation to not provide enough evidence to show whether any of the spices had any effect on the growth or inhibition of histamine accumulation.

	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**
Cumin+ Fish	0.8683	0.8657	0.7586	0.830867
Paprika+ Fish	0.7503	0.8991	0.7317	0.7937
Turmeric+ Fish	0.5109	0.365	0.3538	0.4099
Cumin Only	0.8024	0.7674	0.8837	0.817833
Paprika Only	1.0184	0.9062	0.8886	0.937733
Turmeric Only	0.258	0.4715	0.3918	0.2704
Fish Only	0.3937	0.265	0.2669	0.308533

Table 2. Histamine levels in salmon natively fermented for three days with no added *M. morganii* and without the assay enzyme, histamine dehydrogenase. The absorbance values produced by the salmon experiment contained enough variation to not provide enough evidence to show whether any of the spices had any effect on the growth or inhibition of histamine accumulation.

	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**
Cumin+ Fish	1.0414	1.336	0.8712	1.082867
Paprika+ Fish	0.8172	0.6897	0.7074	0.7381
Turmeric+ Fish	0.4439	1.0778	0.3599	0.6272
Cumin Only	0.777	0.9436	0.8339	0.8515
Paprika Only	1.138	0.9459	1.3546	1.146167
Turmeric Only	0.2867	0.3478	0.6218	0.418767
Fish Only	0.2771	0.2256	0.2195	0.240733

Table 3. Absorbances taken at 565 nm in salmon natively fermented for three days with added *M. morganii* and with the assay enzyme, histamine dehydrogenase. The absorbance values produced by the salmon experiment contained enough variation to not provide enough evidence to show whether any of the spices had any effect on the growth or inhibition of histamine accumulation.

	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**
Cumin+ Fish	0.7698	0.9367	0.9657	0.890733
Paprika+ Fish	0.8215	0.5847	0.6371	0.6811
Turmeric+ Fish	0.5875	0.4426	0.2884	0.4395
Cumin Only	0.9938	0.9444	1.3072	1.0818
Paprika Only	0.8412	1.1203	0.816	0.925833
Turmeric Only	0.2704	0.9645	0.3621	0.532333
Fish Only	0.5011	0.366	0.2747	0.3806

Table 4. Absorbances taken at 565 nm in salmon natively fermented for three days with added *M. morganii* and without the assay enzyme, histamine dehydrogenase.

The absorbance values produced by the salmon experiment contained enough variation to not provide enough evidence to show whether any of the spices had any effect on the growth or inhibition of histamine accumulation.

	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**
Cumin+ Fish	1.3475	0.9843	0.8954	1.075733
Paprika+ Fish	0.9699	0.7531	0.7034	0.8088
Turmeric+ Fish	0.3655	0.406	0.3309	0.367467
Cumin Only	0.971	0.9898	1.3588	1.106533
Paprika Only	0.9076	1.8606	1.6482	1.472133
Turmeric Only	0.998	1.0275	0.3865	0.804
Fish Only	0.2217	0.199	0.2176	0.212767

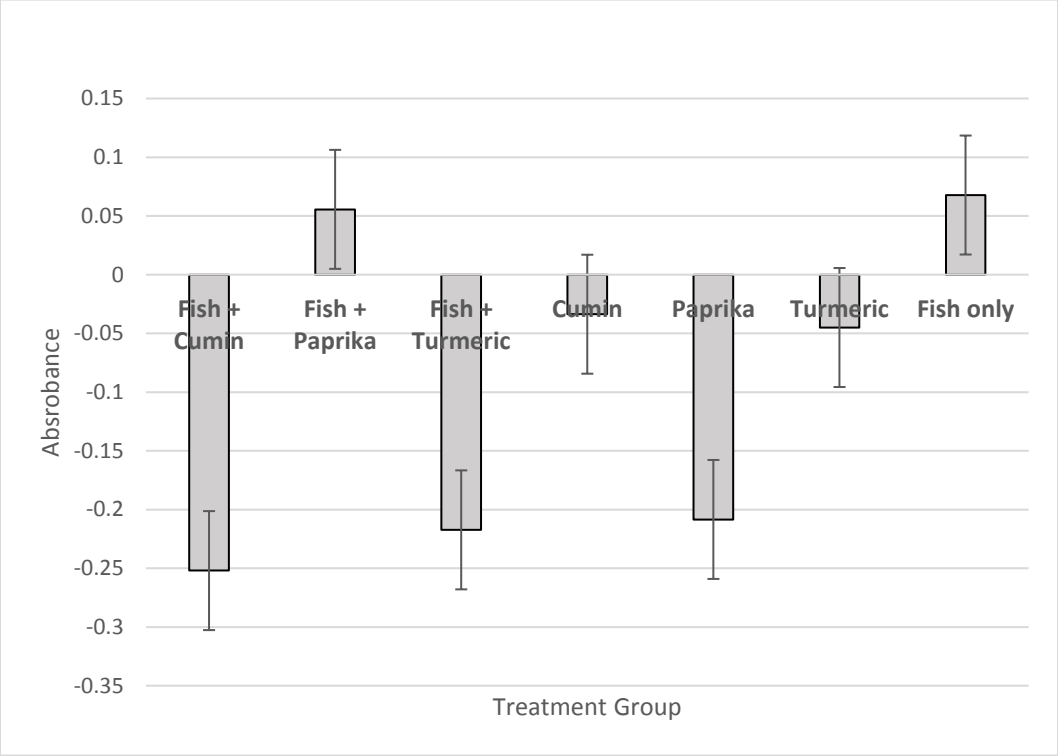


Figure 8. Mean absorbance levels in salmon 3-day natural fermentation. Three independent replicates of salmon were incubated with and without spice, as well as spice alone for three days. A histamine assay was then conducted with and without histamine dehydrogenase enzyme and absorbances at 565 nm taken. Due to the nature of the assay, background level absorbances (-enzyme) must be subtracted from experimental absorbances (+enzyme). This sometimes yields negative mean absorbances. Means are noted as bars with SEM as whiskers. No statistical differences among the treatment groups were found using ANOVA.

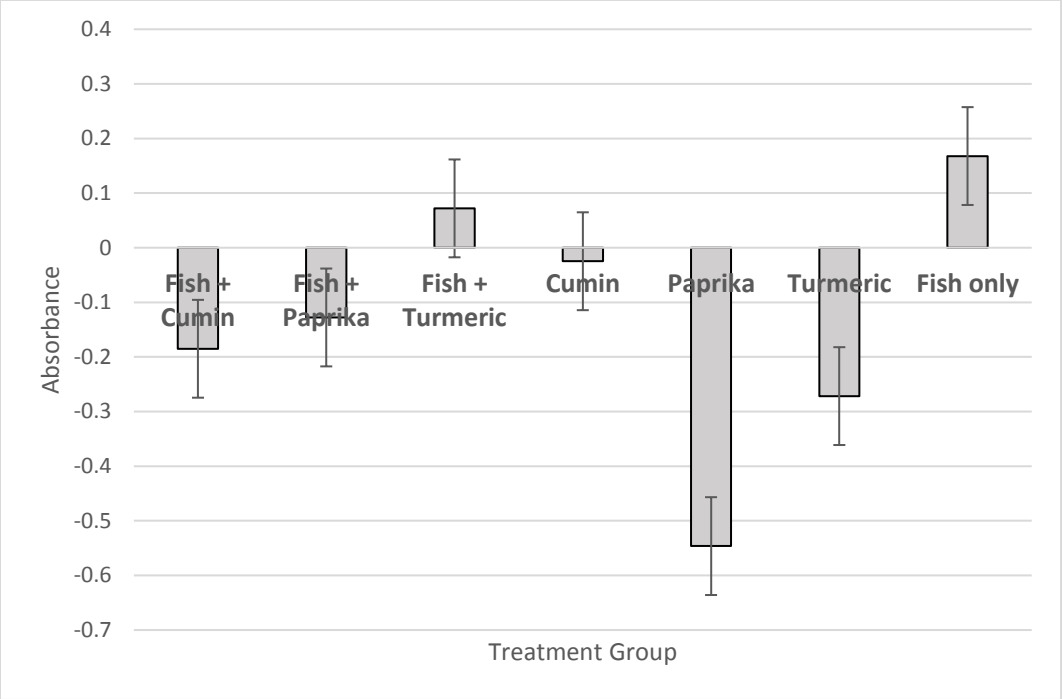


Figure 9. Mean absorbance levels in salmon 3-day assisted fermentation. Three independent replicates of salmon were incubated with and without spice, as well as spice alone for three days following additional inoculation with *Morganella morganii*. A histamine assay was then conducted with and without histamine dehydrogenase enzyme and absorbances at 565 nm taken. Due to the nature of the assay, background level absorbances (-enzyme) must be subtracted from experimental absorbances (+enzyme). This sometimes yields negative mean absorbances. Means are noted as bars with SEM as whiskers. No statistical differences among the treatment groups were found using ANOVA.

While no difference was statistically observed among no spice and spice samples, the low histamine levels in combination with high variability among the replicates might have masked real differences. To establish whether spices might be able to inhibit histamine accumulation, conditions expected to produce extreme levels of histamine were undertaken. Mullet fish, the traditional feseekh fish, was inoculated with *M. morganii* for three weeks with and without individual spices (Table 5-6). None of the spices showed reduction in histamine levels or the inhibition of histamine by *M. morganii* (Figure 9). Compared to paprika and turmeric, cumin had the smallest difference between plus enzyme samples and the minus enzyme samples, even though it was a negative difference (minus enzyme values were larger than plus enzyme values).

Table 5. Absorbances taken at 565 nm in mullet fish natively fermented for three weeks with added *M. morganii* and with the assay enzyme, histamine dehydrogenase. The absorbance values produced by the mullet fish experiment contained enough variation to not provide enough evidence to show whether any of the spices had any effect on the growth or inhibition of histamine accumulation.

	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**
Cumin+ Fish	0.5702	1.0502	0.6547	0.7583
Paprika+ Fish	1.8354	1.3596	0.7369	1.310633
Turmeric+ Fish	0.3847	0.9627	0.2909	0.5461
Cumin Only	1.3586	1.0996	0.9183	1.1255
Paprika Only	1.0702	1.0934	1.9121	1.358567
Turmeric Only	0.4989	0.2259	0.2359	0.320233
Fish Only	0.8971	0.8572	0.8305	0.8616

Table 6. Absorbances taken at 565 nm in mullet fish natively fermented for three weeks with added *M. morganii* and without the assay enzyme, histamine dehydrogenase. The absorbance values produced by the mullet fish experiment contained enough variation to not provide enough evidence to show whether any of the spices had any effect on the growth or inhibition of histamine accumulation.

	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**
Cumin+ Fish	0.9551	0.9402	0.6781	0.8578
Paprika+ Fish	0.7356	0.7077	0.7774	0.740233
Turmeric+ Fish	0.2855	0.2753	0.303	0.287933
Cumin Only	0.855	1.0454	1.8798	1.260067
Paprika Only	1.031	0.9699	1.4828	1.161233
Turmeric Only	0.9564	0.2075	0.2118	0.458567
Fish Only	0.2064	1.1206	0.1969	0.507967

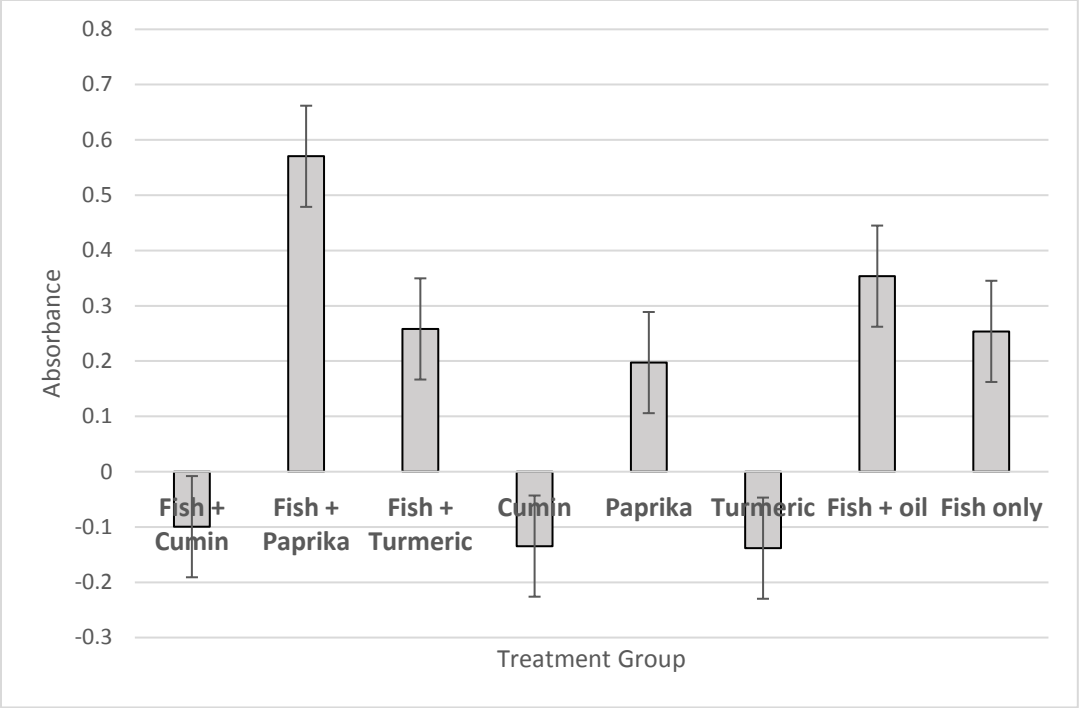


Figure 10. Mean absorbance levels in mullet 21-day assisted fermentation. Three independent replicates of mullet fish were incubated with and without spice, as well as spice alone for three days following additional inoculation with *Morganella morganii*. A histamine assay was then conducted with and without histamine dehydrogenase enzyme and absorbances at 565 nm taken. Due to the nature of the assay, background level absorbances (-enzyme) must be subtracted from experimental absorbances (+enzyme). This sometimes yields negative mean absorbances. Means are noted as bars with SEM as whiskers. No statistical differences among the treatment groups were found using ANOVA.

Conclusions

The high production of histamine in this feseekh dish has caused severe symptoms for individuals who have consumed it (Biji *et al*. 2016). These symptoms can include heart palpitations, difficulty breathing, or even death, in some cases. The goal of this study was to determine whether traditional feseekh spices could affect histamine accumulation either by inhibiting growth of a representative bacterium or inhibiting the production of histamine by the bacterium or by native bacteria. The representative bacterium was *Morganella morganii* because it can be found in this fermented dish and is known to produce histamine. The spices used in this research were cumin, paprika, and turmeric, due to their antimicrobial properties.

Since spices are known to have antimicrobial properties (Rakhi *et al.* 2018), the ability of each spice to inhibit bacterial growth was examined. As seen in the results (Figure 2-4), none of the spices had the ability to prevent the growth of *M. morganii*, as seen in the lack of visible zones of inhibition. Since all the spices failed to prevent the growth of the representative bacterium, the next step was to determine whether the bacterium could produce histamine in laboratory conditions using a spectrophotometric assay. *M. morganii* did produce histamine, however, the levels were too low. To accurately determine whether any inhibition due to spices had occurred, higher histamine levels would be necessary. The absorbance values obtained by the spectrophotometer after using a histamine assay kit were too low to compare to samples with spices.

Due to the very low histamine production by the *M. morganii* in laboratory conditions, more natively fermented experiments were undertaken. Salmon was incubated for three days with and without cumin, paprika, or turmeric and with and

without inoculation of *M. morganii*. Along with being incubated with different spices, some of the samples were also inoculated with *M. morganii*. The absorbances obtained for the salmon experiment contained high variation, which made it difficult to compare the samples to each other to determine whether any of the spices caused the inhibition of histamine in the fish due to *M. morganii*. Using ANOVA, all of the sample absorbances were compared to each other to determine whether there was any statistical difference between any of the data sets. There were no statistical differences found using ANOVA. Due to there not being a statistical difference, it can be concluded that none of the spices had any kind of inhibition effect on the salmon during the 3-day fermentation process. However, the high variability in the absorbance values for the samples made it difficult to compare the samples to each other and any true histamine inhibition caused by the spices may have been masked.

Since the salmon experiment provided preliminary results, it was determined that the next experiment would need to include conditions that resulted in very high histamine production. The next experiment that took place was with previously frozen mullet fish, which is the traditional feseekh fish. The period of incubation for the mullet fish was three weeks and all samples contained the addition of *M. morganii*. Using ANOVA, all of the sample absorbances were compared to each other to determine whether there was a true statistical difference between any of the samples. There was no statistical difference between any of the samples. Due to this, it can be concluded that the spices were not able to inhibit histamine production in the mullet fish by *M. morganii*. The absorbance values obtained for this experiment were also extremely high in variability and any inhibition

due to the spices could have been masked. Cumin seems to show a promise in inhibiting the production of histamine, but there is no strong evidence resulting in this conclusion.

Due to the high variability in absorbances for both the salmon and mullet fish experiments, it can be concluded that there were too many steps that resulted in little variation adding to large variation by the end of the experiments. There were many samples and this variation could have built up during every step, which resulted in very inconsistent data for all of the samples. The high inconsistency in absorbance levels could also have been due to enzymes present in the spices that could be causing them to produce higher absorbance levels than the fish and spice samples. Enzymes present in the spices could also be working against the assay and causing it to exhibit higher absorbances than the fish and spice samples. Due to the strong color of the spices, their absorbance could have also added onto the absorbance values and caused additional variability in the results.

A major problem that was encountered through these experiments was that the bacterial cultures and the fish (both salmon and mullet fish) were not producing enough histamine. This may have been due to using previously frozen fish for both fish experiments, which decreased the levels of histamine present during the fermentation process. Anecdotally, feseekh is made using fresh caught mullet fish that has not been frozen and the guts and fins are usually kept intact with the fish when it is prepared for fermentation (Appendix A). To produce higher levels of histamine in future research, these measures should also be taken to produce extreme amounts of histamine so that histamine inhibition may be seen.

The histamine assay (EnzyChrom™ Histamine Assay Kit; EHIS-100) used in these experiments was not capable of obtaining consistent values due to the strength of colors in the spices. Therefore, other assays or forms of analysis should be used in future research to provide more consistent data. Due to colors of the spices being an issue in this experiment, it may be necessary to use an assay or form of analysis that does not have anything to do with the absorbance of the samples, but the compounds that make up the samples. Therefore, it would have to be a form of analysis that determines the concentration of histamine in the samples, which would use the chemical compounds of histamine, rather than absorbance (Adányi *et al.* 2014). The most direct way of measuring histamine levels in these samples would be through High Performance Liquid Chromatography (HPLC) (Shakila *et al*. 1996). By using this method, the relative amount of histamine in the samples would be obtained through the separation of different components or compounds.

Even though there was no solid conclusion made from this research, these spices should still be used in the making of feseekh. While it is not known whether cumin, paprika, and turmeric can inhibit the production of histamine or the growth of *Morganella morganii* in these experiments, it has been proven over time that spices do have antimicrobial effects on various pathogens (Liu *et al.* 2017) (Shakila *et al*. 1996).

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Appendix

Feseekh recipe

This is an informal recipe that is used by most Egyptians to prepare feseekh, depending on the location. It is passed on in the oral tradition where parents or grandparents instruct as the dish is prepared. This particular recipe was told to Merna Ghobrial by her mother, Sohier Makar.

- Make sure the fish (mullet fish) is first thawed out (if frozen) so that it is easier to work with and use whole fish, not fillets.
- Make an opening in the fish and degut the fish, if not already degutted. Remove all fins off fish. Wash the fish thoroughly. (All insides and fins can be kept on fish to make it ferment faster and have ready to eat earlier.)
- Dry the fish as much as possible using paper towels.
- Use salt to fully rub the fish all over, inside and out. Pack the inside of the fish with salt. There is no known amount of salt, but instincts are used to know when you have used enough salt.
- Different spices, based on preference, are used and applied the same way as salt. They are rubbed on the fish thoroughly and are also packed inside the fish, depending on amount preferred of each spice.
    - Spices include cumin, paprika, turmeric, crushed red pepper flakes, chili powder, and coriander.
- Use a container in which you can tightly pack all the fish closely together and add about one half a tablespoon of canola or vegetable oil for each fish. Place container inside a few plastic bags and place in warm dark area.

- Check on fish in about 20-30 days. Feseekh should be ready when fish is tender to the touch.
- Once ready, fillet the fish and enjoy with lime juice.