Morphological And Molecular Characterization Of Infectious Parasites In Argentine Hake Fish Traded Via Aqaba Port Of Jordan

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Abstract
Various parasites were found in raw fish, and the majority of the scientific literature describes methods for controlling major parasites of concern in the Western World, such as Anisakis parasites (Beldsoe & Oria, 2001; Harvell, 1999). According to archiverecords, the Argentine Hake fish (*Merluccius hubbsi*) was the most parasitically infected fish species (81.8%). In addition, Argentine Hake fish was the most imported fish and had the highest infection rate, violating Jordanian standards when compared to other species of fish infected by parasites.

Thirty-three Argentine Hake fish samples were collected from Jordan's Aqaba port. Total bacterial count and culture for*Salmonella, Vibrio, Pseudomonas, and E. coli* was done. Freshness, Heavy metals, pH, crude protein and lipid, Moisture, Salt,Collagen, Total Volatile Base Nitrogen (TVBN), and Histamine were among the tests performed on those samples. According to thePCR assay, the main parasites isolated from the Argentine Hake fish were Myxosporea kudoa (Kingdom*: Animalia,*

Phylum*: Cnidaria,* Class*: Myxosporem,* Order*:Multivalvulida,* Family*: Kudoidae,* Genus*: Kudoa*). In addition, it was demonstrated that the severity of parasitic infection led to a significant increase in acidity, salt, fat, and water content in the muscle (P>.05).

Furthermore, the severity of parasitic infection led to a significant decrease in protein, collagen, TVP-N, freshness, and bacterial count. Here, parasite density is quantified as the number of parasites per kilogram of fish (>1 parasite/kg fish wt.).

The results of this study suggest that local markets urgently require additional control measures, including the assistance of skilledinspectors. Additionally, it was advised that enough samples be taken from each shipment of fish to allow for a reliable analysis at the specialized labs. Additionally, change the Jordanian standard to "prevent entry of contaminated frozen fish with parasites in the flesh whether it is pathogenic or non-pathogenic," particularly in those parts that deal with frozen fish.

Key words: Fish, Parasites, Argentine Hake fish, and *Myxosporea kudoa*

INTRODUCTION

Due to the widespread consumption of seafood on a global scale, several parasites were found and are now being consideredpotential food hazards.

There was evidence that the parasites *Hyterothylacium* and *Anisakis Contracaecu multipapillatum* could infect mammals and primates, respectively. Additionally, recent climate changes and increased human activity have accelerated the global transport of parasites linked to food, and it is unavoidable that the spread of these species will hasten host-shifts in a way that is difficult to predict.(Harvell, 1999; Porter, 2013)
In addition, the larvae that hatch may infect a small crustacean that may in turn be ingested by a fish such as rockfish,

herring, mackerel, and salmon, which are all of commercial value (Beldsoe & Oria, 2001; Owusu-Apenten & Vieira, 2023). Human being as a result might become infected by eating the infected intermediate host. In addition, these nematodes do not normally mature, butthe worms can migrate from the gastrointestinal tract, becoming embedded in the gastrointestinal mucosa and causing tissue reaction and discomfort like the gastric pain, diarrhea, vomiting(Konecny, 2022; Yanong, 2002).

Other example is the Cestodes, which are tapeworms, and the species of major concern associated with consumption of fish are in the genus Diphyllobothrium. In fact, humans are one of the definitive hosts for such infectious parasites. Salmon is the most common fish that transmits Diphyllobothriasis, although whitefish, trout and pike (Bristow & Berland, 1991; Nguyen, Dorny, Nguyen, & Dermauw, 2021; Norbury, Shirakashi, Power, Nowak, & Bott, 2022) may also transmit it.

Trematode (or flukes) species of concern can be transmitted by the ingestion of seafood may reach sexual maturity in the liver, intestine or lungs of humans and other mammals and some of them can cause serious liver damage (Beldsoe & Oria, 2001; Fong &Chan, 2022) and have been associated with carcinoma of the liver.

Fish parasites are considered a crucial source of concern for consumers of fish in general. This might be because of their possible impacts on the quality and palatability of fish, which could be a justified reason for the reluctance of many people from eating fish or other seafood types.

Citizens in Jordan have raised concerns about fish containing worms or other parasites, particularly those imported through the portof Aqaba. As a result, local consumers' willingness to consume imported fish is still being debated. As a result, it is critical to work in the direction of changing consumers' attitudes toward their willingness to consume fish. This certainly necessitates scientific evidence on the status, conditions, and health of fish entering Jordan via Aqaba port for public consumption.

Our research was primarily concerned with learning more about the genetic makeup of the infectious parasites that are present in Argentine hake fish that enter Jordan. It also placed emphasis on knowing whether these parasites are pathogenic. Additionally, our study looked into any potential impacts that parasites may have on the fish's textural quality.

Study Importance

Our current study provides detailed information for the first time on the various parasites that may be present in the Argentine Hakefish that enters through Aqaba port. It also intends to assess the direct impact of identified parasites on fish quality, such as texture,biochemical constituents, and organoleptic characteristics.

MATERIALS AND METHODS

Study area

The study was conducted in Aqaba, Jordan. Aqaba the only coastal city in Jordan and the largest and most populous city on the Gulfof Aqaba. Situated in southernmost Jordan. Aqaba had a population of 208,000 in 2022 and a land area of 375 square kilometers. Today, Aqaba plays a major role in developing of the Jordanian economy, through the vibrant trade and tourism sectors. Aqaba's strategic location at the northeastern tip of the Red Sea between the continents of Asia and Africa has made its port important throughout thousands of years.

Major part of the current study analysis including all the following parameters in the subsequent section was conducted in the Aqaba International Laboratories (Ben Hayyan). Analyses for genetic codes were performed at the laboratories of Limoges University/France after the process of DNA extraction that was conducted in the laboratories of Jordan University of Science and Technology.

Samples collection

Frozen fish samples that were imported from Argentina to Jordan via Aqaba port were subjected to a thorough investigation at the Ben Hayyan Laboratories, Aqaba.

Another group of fish samples were collected from the local market including different governorates and then examined to determine the effect of parasitic infection on the quality of the fish. A review on parasitic infections of fish that were isolated at Ben Hayyan laboratories were also documented for the period 2014 - 2022 Several tests were conducted on the two groups of fish infected with parasites and fish that were not infected with parasites. These tests are:

Detection of Escherichia coli, Detection of Salmonella spp., Detection of Vibrio parahaemolyticus, Detection of Pseudomonas aeruginosa, Measurement of pH, Total Volatile Basic Nitrogen (TVBN), Near Infrared (NIR) for Fat, Moisture, Protein, Collagen and Salt, Heavy Metals (Cd and Pb), Freshness analysis for the infected and non-infected

fish

Data analysis

Statistical analysis was based on ANOVA and is presented as means. Statistical significance was considered at P-value of 0.05 orless. The data were analyzed statistically using Sigma Stat statistical software version 3.5.

Normality Test: Passed (P = 0.135)
Equal Variance Test: Passed (P = 0.080)

Table (1). t-test analysis between % of Infected and Hake fish collected from local market

Group Name	N	Missing	Mean	Std Dev	SEM
% Infected Fish	7	0	0.134	0.0708	0.0268
% infected Hake	7	0	0.882	0.131	0.0495

Difference -0.748
t = -13.295 with 12 degrees of freedom. (P = <0.001)Power of performed test with alpha = 0.050: 1.000
95 percent confidence interval for difference of means: -0.871 to -0.626

The difference in the mean values of the two groups is greater than would be expected by chance; there is a statistically significantdifference between the input groups (P = <0.001).

Table (2): Statistical analysis of all criteria on Hake fish

Group	N	Missing	25%	75%
pH	33	0	7.010	7.092
APC	33	0	525.000	38000.000
Infected by Parasite	33	0	0.000	2.250
Salmonella	33	0	0.000	0.000
Vibrio Parahaemolyticus	33	0	0.000	0.000
E.coli 0157	33	0	0.000	0.000
Histamine	33	0	0.000	0.000
Salt	33	0	1.550	1.700
Total Protein	33	0	17.375	17.942
Moisture	33	0	78.942	80.500
Total Fat	33	0	2.260	3.060
Collagen	33	0	1.780	2.030
TVPN	33	0	14.040	32.000
Freshness	33	0	1.600	3.050

H = 436.394 with 13 degrees of freedom. (P <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is astatistically significant difference (P<0.001)

To isolate the groups that differ from the others the study used a multiple comparison procedure.

Polymerase chain reaction (PCR)

Multiple PCR assays were used to examine the genetic characteristics or bar-coding of parasites and fish. Prior to PCR techniques, DNA was extracted using a commercial kit (DNeasy® Blood & Tissue, Qiagen. Germany) from the investigated parasites and the investigated fish.

The parasite responsible for the infection of the Argentine Hake fish was identified as Myxosporean based on morphologicalcharacteristics as Fig.I.

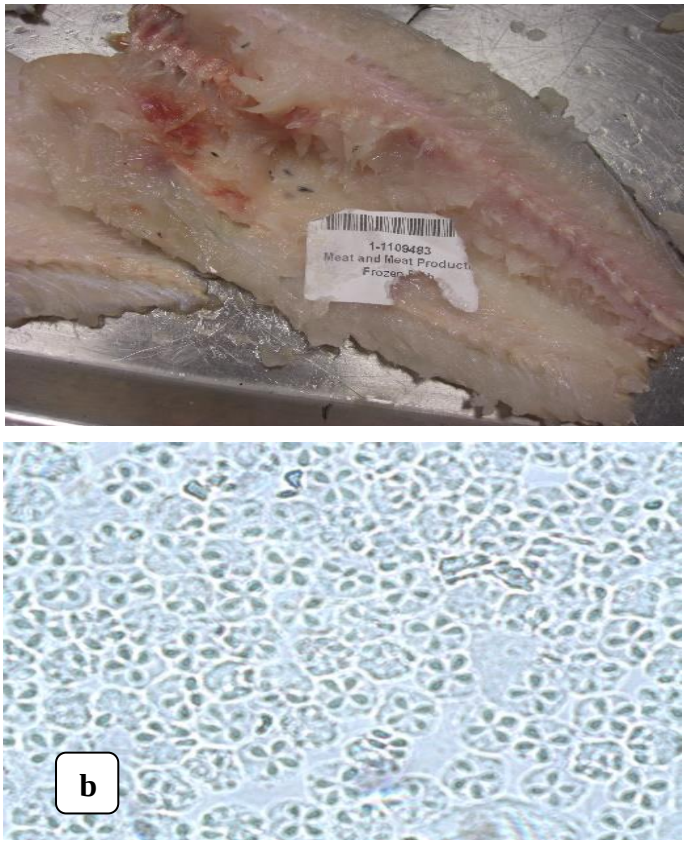

Figure 1. a. Hake fish infected with Kudoa Black pseudocysts), **b**. Black pseudocysts in hake fish under microscope (1000X magnification) (note: all scale bars in cm).

Collected parasites were homogenized using a tissue homogenizer. About 25 mg of the homogenized tissue were placed into a 1.5 ml micro-centrifuge tube. 180 µl of animal tissue lysis buffer (ATL) was added along with 20 µl proteinase K enzyme and mixed thoroughly by vortex. The mixture was then incubated at 56°C in shaking water bath for 3 hours. Additional quantity of 200 µl of Lysis buffer (AL) were added to each sample and then mixed thoroughly by vortex. An amount of 200 µl 96% ethanol was added tothese aliquots and mixed again by vortex. The mix were then pipetted into the DNeasy mini spin columns and placed in a 2 ml collection tubes. Tubes were centrifuged at 8000 rpm (Biofuge fresco: Max. radius 8.5 cm) for 1 min. The flow-through fluid and collection tube were discarded and the DNeasy mini spin column was placed in a new 2 ml collection tube. 500 µl of wash buffer (AW1) were added to tube and centrifuged at 8000 rpm (Max. radius 8.5 cm) for 1 min. The flow-through fluid and collection tubewere discarded and the DNeasy mini spin column was placed again in a new 2 ml collection tube. Another 500 µl of Wash Buffer AW2 were added, and then centrifuged for 3 min at 14,000 rpm (Max. radius 8.5 cm). At this stage, both the flow-through and collection tube were discarded. At final stage, the DNeasy mini spin columns were placed in a clean 1.5 ml microcentrifuge tube together with 100 µl Elution Buffer (AE) were added directly onto the DNeasy membrane that was incubated at room temperature for 1 min followed by centrifugation for 1 min at 8000 rpm (Max. radius 8.5 cm) to elute the DNA. In order to measure the concentration of the eluted DNA a Nano Drop (ND 1000) spectrophotometer device was used(Knot, Zouganelis, Weedall, Wich, &Rae, 2020).

The DNA extracts were subject to specific PCR reactions for both parasite and fish muscle tissues. PCR reactions were composed of 12.5 µl of PCR master mix solution, 1 µl of each primer (10 pmol) (eurofins genomics, Germany) (Table III), and 7.5 µl water were added to each sample (Table IV). The final volume of each PCR reaction was 22 µl and 3 µl from the extracted DNA. PCR reactions were performed according to the following program:

Table 4; PCR reactions conditions

94 °C	5 min	
94 °C	30 sec	X 35 cycles
52 °C (for parasite samples) or 56 °C (for fish samples)	30 sec	
72 °C	45 sec	
72 °C	5 min	

After amplification, 10 µl of the PCR products were run in a 1.5 % agarose gel electrophoresis and visualized on an UV transilluminator (Bio-Rad Molecular Imager Gel Doc™).
Note that bands of a low molecular weight move very quickly through the gel (Fig. III) while high molecular weight bands move very slowly (Fig. II + III).

Table 4: PCR primers used in the present study including names, targets, sequences and expected sizes

Primers name and targets	Primers sequences
Mer – hub – CR –AS (Fish)	AGT TCA GTA AGG TCA AGG GT
Mer – hub – COI –S (Fish)	GCA TAGT CGG AAC AGC CCT A
Mer – hub – COI –AS (Fish)	TGC TGG TAT AGG ATG GGG TC
Mer – hub – CR – S (Fish)	GCT ACT CTT ACC TTC AGC CCT
KU – 18 S – R1 (Parasite)	CTA ACG CCC TCA AAT GTT CC
KU – 18 S – F1 (Parasite)	CAA GGT GGT AAC GGG TAA CG
KU – 18 S –R2 (Parasite)	TCT GGA CCT GGT GAG TTT CC
KU – 18 S –F2 (Parasite)	CTA CTG GAG GGC AAG TCT GG

In order to run the mix on the sequencing machine the following protocol was followed; 400 µl of 5% sephadex were placed on the 96 well plates. The plate was then centrifuged for 3 min at 2500 rpm (Max. radius 8.5 cm) to create a column of sephadex. By centrifugation, the water was separated at the bottom of sephadex after which about 20 µl of sequence product was taken and placed in the center of sephadex column and centrifuged again for 3 min at 2500 rpm (Max. radius 8.5 cm). This was followed when the 96 well plate was placed into the thermocycler for 20 minutes at a temperature of 94 °C leaving the lid open, then the plate was placedon ice for 3 min. This was followed when the liquid from the plate were transferred into a special plate with a rubber cover at whichit was placed in a sequencer for 3 hours in order to get the final results that were recorded accordingly. By the completion of sequencing, the results were viewed into the sequencer software and analysis was performed using the Blast software following the link: http://blast.ncbi.nlm.nih.gov/Blast.cgi

RESULTS

This study provided an accurate identification of parasites that are incorporated in flesh of Argentine Hake fish via Aqaba port. Since this is the first time to follow infectious parasites in fish of commercial value, such identification and documentation are considered innovative and of great value to fish industry and fish health in Jordan. Phylogeny and genetic information of parasites support the assumption that parasites affect the fish in terms of both healthand quality.

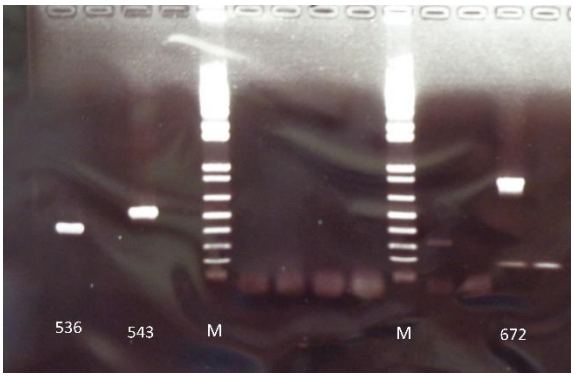

Figure 2. Movement of the DNA bands through the gel, where molecular weight bands 543, 536 and 672 bp.

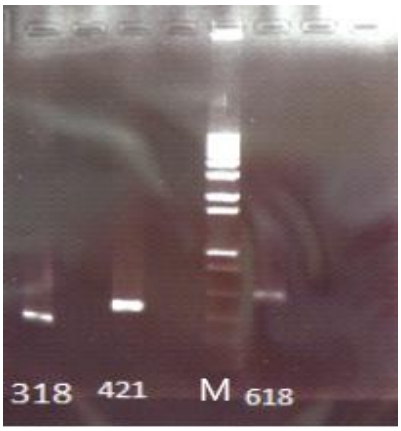

Figure 3. Movement of the DNA bands through the gel, where molecular weight bands 318, 421 and 618 bp.

Varying characteristics of the examined fishes were observed as (Table VI);
The pH values of non-infected fish were ranging from 7.03 to 7.17 while it was from 6.88 to 7.08 in infected fish (Fig. IV).

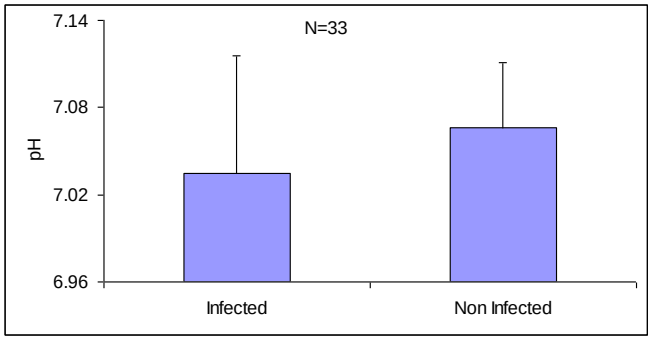

Figure 4. pH values in the examined fish

The APC value was ranged from 5,000 to 90,000 colony forming unit (cfu) in non-infected fish while it was from 200 to 4,000 cfuin infected fish (Fig. V).

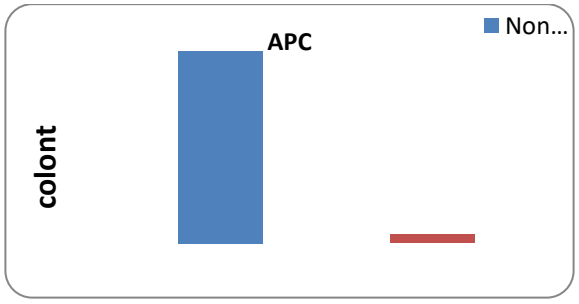

Figure 5. APC values in the examined fish

In addition, average total fat in infected fish was 2.93 g/100g while in non-infected were 2.84 g/100g (Fig. VI).

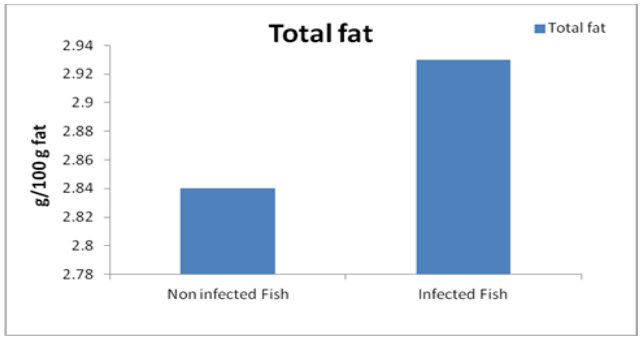

Figure 6. Total fat in the examined fish

Moreover, the total protein was within the amount of 17.75 g/100g in infected fish while it was in non-infected 17.85 g/100g (Fig.VII).

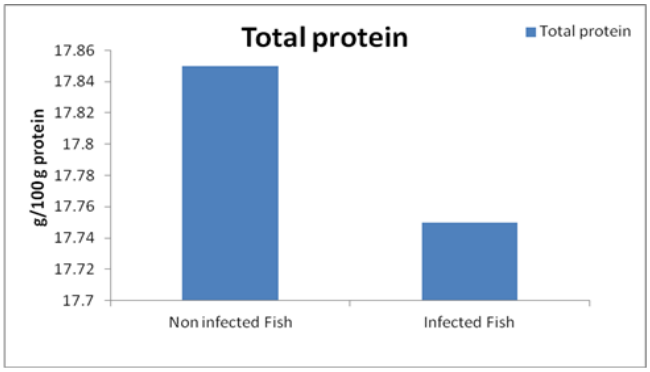

Figure 7. Total protein in the examined fish

Average collagen in infected fish exhibited a value of 1.97 g/100g, but this value was in non-infected 1.80 g/100g (Fig. IIX).

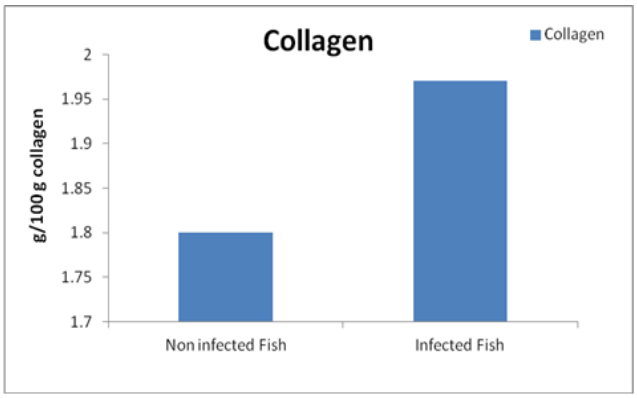

Figure 8. Collagen values in the examined fish

In addition, the average of TVP-N test in infected fish 14.04 g/100g while in non-infected were 16.38 g/100g (Fig. IX).

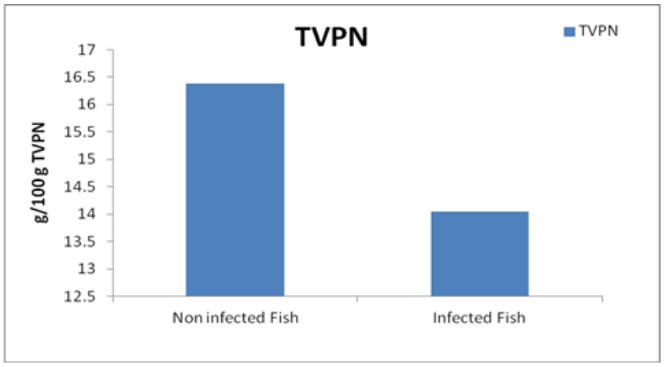

Figure 9. TVPN values in the examined fish

Moreover, the average of freshness value in infected fish was found 7.81 while in non-infected were 9.70 (Table VI).

Table 5. Organoleptic Chart by Using the Distell Freshness Meter

Organoleptic	Raw odour description	Cooked flavour description	Taste panel score	Freshness Meter score	Days on ice	EC Grade	State of spoilage
Non-infected Fish	Bread, malt, beer, yeasty odours	Insipid, no flavours	6	10	~ 11	B	Decrease in Freshness
Infected Fish	Lactic acid, sour milk, or oily odours	Trace of "off" flavours, some sourness but no bitterness	5	8	~ 14	B	↓

Table 6. Summary of results for some tests

Test	Non infected Fish	Infected Fish
APC	5,000 to 90,000 cfu	200 to 4,000 cfu
Total fat	2.84 g/100g	2.93 g/100g
Total protein	17.85 g/100g	17.75 g/100g
collagen	1.80 g/100g	1.97 g/100g
TVP-N	16.38 g/100g	14.04 g/100g
Freshness	9.70	7.81

The parasite was characterized as a non-pathogenic but certainly, it negatively influenced the quality of fish; hence might reduce theeconomic and nutritional value of these fish. Also, Argentine Hake represented the highest fish percentage in term of quantity. At the same time, it was found among the highest in ratio (88%) of the infected parasite.

In addition, isolated parasites from frozen fish that are imported via Aqaba port were found dead and have no direct harm to humans, the most effective means of killing the parasites are either freezing or heat inactivation (Rausch & Adams, 2000; Rausch, Adams, & Margolis, 2010). However, the parasites were found as cysts in muscle tissue, which make them mucoid as Myxosporea Kudoa as Fig. X.

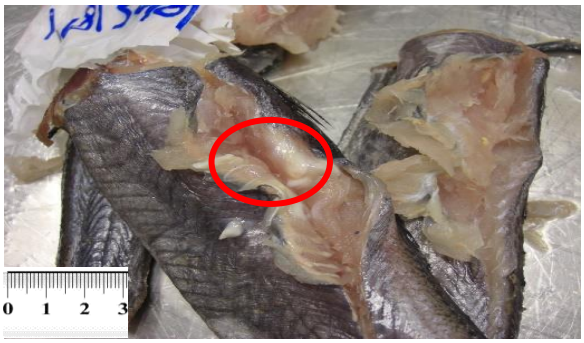

Figure 10. Hake fish infected with Kudoa (white pseudocysts)

Consequently, their existence reduces the commercial value of the infected fish; the most serious impact of Kudoa is the spoilage of fish fillets as it causes extensive post mortem myoliquefactive necrosis (Kristmundsson & Freeman, 2014) (Burger & Adlard, 2013; Iwashita et al., 2013; Kawai et al., 2012; Yokoyama & Itoh, 2005). The effect and progress of this, shortly after fish death the thin plasmodial membrane degenerates exposing the spores to the muscular tissue enveloping the parasites. The presence of the spores causes this muscular envelope to necrotise with a subsequent liberation of numerous spores into the surrounding muscular tissue. The presence of large white pseudocysts also reduces the marketability of the product (Kristmundsson & Freeman, 2014), then considerably reduce the commercial value of the fish species they infect (Burger & Adlard, 2013; Iwashita et al., 2013; Kawai et al., 2012; Yokoyama & Itoh, 2005) . Furthermore, these fillets are known to be undesirable to consume as they disintegrate during cooking, as has been reported with K. thyrsites (Kristmundsson & Freeman, 2014; Langdon, 1991)

No studies have been made on Kudoa infections in the past, which may be because it is not considered suitable for human consumption. It is commonly called the “jelly cat”, due to its jelly-like muscle, and in most cases is thrown back into the sea when caught (Kristmundsson & Freeman, 2014). Post mortem myoliquefaction became evident and many of the fillets proved unfit for human consumption. In 2008, the farming ceased in wolffish culture plant in Iceland and all fish were slaughtered. There may have been multiple reasons for the termination of wolffish culture in Iceland, of these was the negative impact of Kudoa islandica which certainly played a significant part for such termination. Wolffish aquaculture in Norway has also largely ceased (Kristmundsson & Freeman, 2014; Le François et al., 2021) due to the recent discovery of Kudoa, which was the most causative agent of food poisoning for humans .(Hoai, Nhinh, Giang, Senapin, & Dong, 2022; Iglesias, Rangel, Fernández-Vázquez, Santos, & García-Estévez, 2022; Iwashita et al., 2013; Kawai et al., 2012; Li et al., 2022; Matsukane, Sato, Tanaka, Kamata, & Sugita-Konishi, 2010; Snyder, 2022)Therefore, the potential effect of muscle-infecting Kudoa species upon food safety and public health should be considered(Kristmundsson & Freeman, 2014). Our results showed that there is a negative correlation between the number of parasites and the bacterial count in our examined fish. This outcome was expected as a result of proteolytic enzymes released by the marine myxosporean parasite, Kudoa spp, after the death of the fish, which was consistent with what is known from previous studies (Henning, Hoffman, & Manley, 2013; Hoai et al., 2022).

DISCUSSION

Statistical analysis results demonstrated that there was significant negative correlation between pH value and the number of infected fish (- 0.208) and this could be explained due to the intensity of parasite infection an increase in the flesh acidity that might be as a result of parasites metabolism that affect the pH levels due to the acidic by products produced during the infection period. Moreover, the pH decreases due to anaerobic glycolysis, which generates lactic acid(Papanicolaou, 2013; Possemiers, Vandermosten, & Van den Steen, 2021) . This decrease apparently triggers a processing of the enzyme resulting in post mortem muscle proteolysis the case which is stable in the living fish (Kristmundsson & Freeman, 2014) . Therefore, reducing the amount of glycogen in the muscle fish, prior to harvesting, was considered a possible way of reducing the post mortem effect of K. thyrsites(Funk et al., 2008). Also our statistical analysis results demonstrated that there was significant positive correlation between salt value and the number of infected fish (0.0252) and this could be explained due to intensity of parasite infection caused a significant decrease of the salt content in the muscle ($P < 0.05$). Moreover, our statistical analysis results demonstrated that there was significant negative correlation between total fat and the number of infected fish (- 0.205) and this could be explained due to depletion of total lipids, as well as our statistical analysis results demonstrated that there was significant negative correlation between total protein and the number of infected fish (- 0.188) which were decreased (Abdelsalam et al., 2022; Funk et al., 2008) . Furthermore, our statistical analysis results demonstrated that there was significant positive

correlation between moisture and the number of infected fish (0.0495) intensity of parasite infection increased significantly the moisture content (P <0.05). As well as our statistical analysis results demonstrated that there was significant negative correlation between collagen and the number of infected fish (- 0.135), as the number of parasites while their collagen ratio was decreased. However, our statistical analysis results demonstrated that there was significant negative correlation between TVBN values and the number of infected fish (- 0.116) and this infected fish with parasite was influenced negatively on TVBN values as(F. Giarratana et al., 2014; M. S. Giarratana & Mariani, 2014; Kristmundsson & Freeman, 2014; Mirzaei et al., 2020; Rathod, Nirmal, Pagarkar, Özogul, & Rocha, 2022; Santoro, Palomba, Alburqueque, & Mattiucci, 2022; Trabelsi et al., 2021). In addition, our statistical analysis results demonstrated that there was significant negative correlation between freshness and the number of infected fish (- 0.239) intensity of parasite infection decreased the fish freshness and organoliptic test parameters. More over our statistical analysis results demonstrated that there was significant negative correlation between total microbial count and the number of infected fish (- 0.107). In addition, our statistical analysis results demonstrated that there was no significant correlation between salmonella, E.coli, Vibrio parahaemolyticus and histamine and the number of infected fish and this explained due to all previous values were zero. Finally, the most imported fish type that enters Jordan via Aqaba port is Argentine Hake and Mackerel fish (64%). These fish are actually the cheapest in world's markets but was also found to be the most infected fishes with parasites presented in this study.

CONCLUSIONS

Therefore, our study would recommend increase control over the local markets by adding more inspectors/ food analyst to imported fish in general and to this type of fish in particular. In addition, assign personnel (veterinarians) with good experience in food analyses and to develop more food laboratories in other locations in the country. Moreover, adequate fish samples for analysis are needed in order to represent fish/ shipment for more accurate and confident results. Furthermore, educate the consumers on fish nutritional value and the quality of Hake fish. Moreover, Jordan Institution for Standards and Metrology (JISM) is requested to amend the Jordanian standard that is in charge of frozen fish to be in the following order "prevent entry of contaminated frozen fish with parasites in the flesh whether these are pathogenic or non-pathogenic regardless of the number of the inspected parasites". So Jordanian standard generally does not allow the importing of fish from any country announced to be infested with epidemic diseases. Thus, our study recommends announcing that Argentina is infested country with epidemic parasitic disease in fish, hence banning any fish imports from this country. in addition this study recommend encouraging the use of a state-of-art method for identifying parasites utilizing molecular biology that often replace traditional approaches because of their rapidity, sensitivity, ease of use and accuracy.

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