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Contamination of Fish Feed with Pathogenic Organisms: Implications on Fish Diseases in Aquaculture Systems

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ABSTRACT

Tracing contamination to its ultimate source is considered difficult as multiple factors affect stable microbial community in culture water. Feed, covering over 60 percent of production cost although less considered, could represent a significant source of fish diseases, the major constraint in aquaculture production. The present study based on the sensitivity of quantitative Polymerase Chain Reaction (qPCR), and sequencing analysis was undertaken to determine the microbial load in fish feed and its potential to cause disease outbreaks in aquaculture farms in Ghana. From the general screening of a total of 30 feed samples, 90% were positive for bacterial contamination, 70% for fungal contaminations, with 40 % showing multiple contaminations with both bacterial and fungal pathogens Three important bacteria pathogens (Streptococcus iniae, Streptococcus agalactiae and Staphylococcus aureus) implicated as major causes of fish diseases occurred at prevalence rates of 89 %, 60 % and 60 % respectively in the fish feed and with average concentrations(Ct values) of 28.39, 40.78 and 34.26. Additionally, sequencing analysis based on the 16S rDNA gene of bacteria and at less than 96% identity, revealed the presence of three different genus of bacteria, and six different species, representing a profile of over 50 different strains of bacteria from the Sphingomonas genus. A positive correlation (0.667) existed between the prevalence rate of bacteria species in fish feed and that in diseased fish ($P<0.05$). Also, the prevalence rate of bacteria isolated from the gut of fish (26.09%) were significantly ($P<0.05$) similar to that from diseased portions (26.89%). Although mode of transmission of most infections are known to be largely through the water in which the fish are submerged, prevalence rate of S. iniae (32 %) and S. agalactiea (24%) were significantly($p<0.05$) lower in water samples than that in the feed (89%, 60%) respectivelyIn addition, Staphylococcus aureus which was not isolated from the culture water samples at all, occurred at a prevalence rate as high as 60 % in fish-feed. This thus confirms contamination of fish-feed as a significant pathway for entry of pathogens. Evaluation of microbial quality of fish feed as a routine practice on farms should therefore be enforced.

***Keywords**: Fish feed, Fish diseases, Microbial Quality, qPCR, DNA*

INTRODUCTION

Towards achieving food security, the safety and good health of fish and consequently on consumers cannot be compromised. Thus, aquaculture production, focused on increasing consumption of fish using methods that minimizes risks to fish and public health is highly critical. The current practice of using alternative sources of feed to mitigate against the globally high cost of feed (FAO, 2017; Bimbo & Crowtber 1992) coupled with the desire to obtain fast growing fish that reaches marketable size within the shortest possible time per the increase demand, raises concerns on the effects on fish through microbial contamination of fish feed.

When fish are reared under confinement at high densities where free foraging on natural feeds are impossible, they must be provided a complete diet (Craig & Helfrich 2018). Proper nutrition particularly, during larval stages is critical towards successful and sustainable hatchery operations. The ingredients used in the formulation of feeds may vary regionally, and per fish species, but typically it constitutes, fishmeal (16 percent), peanut meal (24 percent), soybean meal (14 percent), rice bran (30 percent), broken rice (15 percent) and vitamin/mineral premixes (1 percent) (Somsueb 1994). Feed manufacturers put mixtures of these feedstuffs and feed additives into a usable form with the aim of increasing profits of animal production by increasing the nutritional value of the mixture of feedstuffs. Most fish feeds must be pelleted, water stable, and generally made to float (extruded feeds) on the water surface, for efficient utilization by fish. However, due to the limited and high cost of raw materials as well as the equipment for mixing, manufacturing cost of fish feed is fundamentally high. The use of alternative sources of feeds such as trash fish, animal by-products and grain by-products, or reverting to the use of single ingredient supplementary feeding regimes, as well as reduced feeding frequency and ration, have become a major consideration and as such, a major practice in most farms, as a means of mitigating against the high cost of feed (FAO, 2019). A much less expensive feed is the use of fresh feed like poultry viscera, fish gills and viscera, kitchen refuse and bread which are directly dumped into ponds upon arrival at the farms (New 1987). This has been found to be very common among catfish farms. Several on-farm experimental studies have been reported using various additives in feed such as the use of Jatropha *curcas* Kernel Meal (Workagegn et al. 2013**;** Kumar et al. 2010), Chicken offals and spoiled eggs (Budiati et al. 2015), moringa (Afuang et al. 2003, Ritchter et al. 2003), to improve palatability and growth performance. Adulteration of fish feed with anti-nutritional factors and toxic chemicals like melamine, as well as nutritionally unbalanced diet, have also become widespread for its potential benefit in cutting down the cost of feed (Source: www.naturalnews.com/025836.html). All the various interventions against the increasing cost of feed can compromise the health and welfare of fish, reduce their immune response, the productivity and production, and consequently affect humans, the main consumers (FAO, 2019).

As a preventive measure for disease outbreak on fish farms, the rule of thumb is to avoid the introduction of pathogens to fish, often achieved through proper husbandry practices. This is critical as the devastating effect of the environmental impacts on aquaculture production can sometimes offset the potential benefits. Hence, in recent times, concerns on fish feed quality and effects of fish feed on the aquaculture water environment have been intensified as reported by several authors (Yildiz et al, 2017; Ballester-Moltó et al. 2017; Ballester-Moltó 2016; Huang et al. 2016; Edwards, 2015; Amirkolaie, 2011). However, most discussions on fish feed usually focuses on the effects of the released macronutrients like nitrogen and

phosphorous from uneaten feed or fish excretion on water quality (Wang et al. 2013; Akhan, & Gedik, 2011) and hardly considers the impact of the microbial quality of the feed itself being introduced directly. Highlighting on the fact that most research has generally not considered the quality of fish feed (Wu et al.2012; Rahman et al. 2008), a recent study which showed that different fish feed qualities affect or stimulates algae growth, was again based on the nutritional quality of feed, regarding differences in nutrients concentrations, but was undertaken under experimental settings, with effects limited to algal growth (Kong et al. 2020) and no linkage to fish diseases.

Fish feed, whether commercially made or on-farm made can usually get contaminated with chemicals through additives and microbes through handling, improper storage and variations in heat and humidity conditions. Micro-organisms are ubiquitous and can be found everywhere, consequently under optimum conditions they can proliferate. Moisture in these highly nutrient rich feed compositions, usually purchased as bulk feed in truckloads and stored for long days in outside barns, highly facilitate microbial load. Aquafeeds can therefore serve as a carrier for a range of microbial contaminants such as molds, mycotoxins and bacteria (Maciorowski et al. 2007).

Fish and fish products have therefore been reported as vehicles of food-borne bacterial infections in humans (Novotyn et al. 2004, Hastein et al. 2006, Efuntoye et al. 2012). Ensuring that fish feed is free of contaminants should possibly help reduce fish diseases as well as human foodborne illness, currently on the rise. However, amongst the deterministic and stochastic factors that affect stable microbial community in culture water and in disease outbreaks, direct input from exogenous feeding is less considered. Hence, reports on public health crisis with contaminants linkage to fish and to humans via contaminated fish feed do exist scantly in the scientific world. Thus, as part of advancing efforts in fish health management, this study was undertaken with the focus on utilizing molecular techniques for its advantage of rapidity, accuracy and sensitivity in detecting and quantifying microbial contaminants of significance in fish feed as an important component that describes the quality of feed. The study targeted various aquaculture farms in Ghana and drew potential linkages between microbial quality of feed and its impact on fish health and humans through consumption.

EXPERIMENTAL

Study Area and Sample Collection

A maximum of five varying sizes; powdery to 4.5mm, (Fig. 1) of the most predominant commercial fish feed used in Ghana were aseptically collected from six selected aquaculture farms in three regions of Ghana. Feed samples taken were typically of those under storage within 1 to 6 months. Tilapia samples (60 each of healthy and diseased) as well as a total of 6 water samples, and a replicate of five per farm, were also taken from each farm, kept on ice and transported to the laboratory for analysis. Observational guide as well as semi-structured interview guide, was used to solicit for information from each farm regarding general information on fish feed, including types of feed, mode of feeding, storage procedures and disease conditions.

A. 4.5mm	B. 4.0mm	C. 3.0mm

D. 2.5	E. 0.6mm	C. powdery

Figure 1. Images of different size ranges of fish-feed obtained from various aquaculture farms in Ghana.

DNA Extraction and Polymerase Chain Reaction Amplifications.

For each size range of feed sample, 20 g were taken, dissolved in 100 ml of distilled water and then filtered (Austin & Austin 1999). A piece of the air-dried filter paper containing the filtrates, was cut and used as starting materials for DNA extraction. Various tissues of the fish including the liver, kidneys, skin washes (sw), gut contents, diseased portions (dp) were also prepared for analysis after clinical examination and dissection following standard protocols (Noga, 1999). Fifty (50) mg of the fish tissues were used for DNA extraction whiles skin washes, and guts contents were dissolved in 100 ml of distilled water and 100 ml each of the water samples, all filtered for DNA extraction. Following manufacturer's instructions, the Qiagen DNA extraction protocol was used in extracting DNA from all samples and then stored at -20 degrees for subsequent use in PCR amplifications.

All DNA samples were screened for the presence of bacterial and fungal pathogens using the U63/1378 /U_1378R and ITS1/ITS4 universal primers (Table 1), that targets the 16S rDNA gene of bacteria and 18S rRNA gene of fungi respectively. Using specific primers as listed in table 1, three species specific bacteria (*Streptococcus iniae*, *Streptococcus agalactiae*, S*taphylococcus aureus)* were also isolated for its importance in fish disease outbreaks. The PCR reaction protocol had a total reaction mixture of 10 µ l: 5 µl of Syber Green enzyme, 10 µM of 0.3 µl of each primer set, 2.4 µl nuclease free water and 2 µl of DNA. The cycling conditions had an initial denaturation of 94^{o}C for 3mins followed by 30 cycles of 94^{o}C denaturation, (50^{o}C annealing for bacteria, 54^{o}C for fungi and 55.1^{o}C for a

multiplex involving all 3 specific bacteria), all at 45 seconds each and a 72 ^{o}C extension for 1 minute. A final extension was set at 72 ^{o}C for 5 minutes. The reactions included positive and negative controls, where double distilled water and pure bacteria colonies (*Streptococcus*, *Staphylococcus spp.* isolated from diseased Nile tilapia by Microbiology laboratory of the Council for Scientific and Industrial Research-Water Research Institute (CSIR-WRI), Accra, Ghana) respectively, were used as a measure for quality and accuracy of protocol. Amplified PCR products were visualized by 2% agarose gel electrophoresis. Thus, PCR products including 100 bp DNA ladder were loaded in the wells of the gel and run in a 1x TBE buffer containing ethidium bromide by applying an electric of current100Volts for 45minutes. Specimens showing bands against the standard ladder at the expected fragment sizes were recorded as positive for their respective species. Samples were also sequenced using Sanger Sequencing method (Sanger et al., 1980) at South Africa through Inquaba Biotec Company Ghana, to confirm specificity of target species and pathogenicity of strain. It also served as basis for identifying cell viability (Jamil et al., 1993), as detection of longer intact DNA sequences correlates more closely with viability than shorter sequences (McCarty and Atlas, 1993). Sequences were BLAST (Basic Local Alignment Search Tool) analyzed at NCBI GenBank. (National Centre for Biotechnology Information) (http://blast.ncbi.nlm.nih.gov) for confirmation of pathogenic strain of targeted species. Thus, sequencing analysis which are equally used in current research efforts were used here to obtain a profile of pathogens per their percentage of identity which was set at less than 96%.

Table 1: Primer sequences with their expected fragment sizes used for traditional PCR

Organism	Primer name	Sequence (5'-3')	Size(bp)	Ref
Universal Bacteria	U_63F	CAGGCCTAACACATGCAAGTC	1300	Marchesi *et al*., 1998
	U_1378R			
Universal Fungi	ITS1	GGGCGGWGTGTACAAGGC	540	Noveriza et al., 2020
	ITS4	TCCGTAGGTGAACCTGCGG		
		TCCTCCGCTTATTGATATGC		
S. iniae	S.INIAELOX	AAGGGGAAATCGCAAGTGCC	1020	Mata *et al*., 2004
	S.INIAELOX	ATATCTGATTGGGCCGTCTAA		
S. agalactiae	S.AGA F	GAGTTTGATCATGGCTCAG	220	Hu *et al*., 2019
	S.AGAR	ACCAACATGTGTTAATTACTC		
S. aureus	S.AUREUSF	AACTCTGTTATTAGGGAAGAACA	700	Velasco *et al.*, 2015
	S.AUREUSR	CCACCTTCCTCCCCGTTGTCACC		

Quantification of Pathogens (qPCR)

A real-time PCR assay was developed for improved detection and quantification of pathogens in all samples collected for analysis, drawing from the associated advantage of sensitivity, specificity and rapidity. The quantification analysis was again to confirm the viability of isolated cells, in the absence of the culturing method. The qPCR assay was performed and monitored using the VERSANT kPCR Molecular System AD, SIEMENS (Stratagene), with SYBR Green Master Mix. For definitive amplification of the 16S rDNA region of *S. iniae S. agalactiae*, and *S. aureus* species-specific Forward (F) and Reverse (R) primers, Sin-F (5′-TTTAGTGAGGTCCTCGGATCA-3′) and Sin- R (5′GATCCTTCCGCAGGTTCAC-3′), Saga - F (CATTTGCGTCTTGTTAGTTTTGAG) and Saga-R (GGAGCCTAGCGGATCGA), S. aureus Fq (5′-AAGCCTTGCAGGACATCTTCA-3′) and S. aureus Rq (5′-GCCGCCAGTGTGATGGATAT-3′) respectively were used. The qPCR amplification was performed in a total volume of 20 µL containing 2 µL of each template DNA, 1 µL of 10 pmole primers, 7 µL nuclease-free water, and 10 µL SYBR Green I master mix. qPCR assays were carried out using a standard program and cycling conditions with an initial denaturation step of 5 min at 95 °C, followed by 45 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. A melting curve analysis was included at the end of every program from 95 °C to 65 °C at a rate of 0.1 °C to assist in data analysis. Quantification cycle (Cq; CFX96) and crossing threshold (Ct) values were calculated automatically using instrument software. All samples were analyzed in triplicate as a measure for quality.

Specificity and Sensitivity of real-time PCR Amplification

All primer sets were tested for specificity, sensitivity, optimal annealing temperature and primer efficiency as previously described (Bustin, 2010; Bustin et al., 2009). The Real-time PCR assay specificity was optimized and validated using appropriate total DNA extracted from other bacterial species other than the target species, and no template controls (ultra-pure water) in the reactions. The sensitivity of the qPCR assay and quantification assays for construction of standard curves and quantification of samples was evaluated using a 10-fold serial dilution of purified DNAs from viable streptococcus cells (26.3×10^6 to 1 ng/lL) with concentrations calculated using the viable cell plate count method.

Statistical Analysis

Prevalence rates and other numerical data were estimated by Excel, 2019. Multivariate analysis (ANOVA) was used to test the mean significant difference among variables, that is, prevalence rates of bacterial occurrence among feed samples and the various samples analyzed. Pearson's Correlation co-efficiency was used to determine the correlation between prevalence rate of pathogens in feed and fish. The significance level was set at $P < 0.05$. Sequenced results were blast analyzed at NCBI. They were then used in constructing a phylogenetic tree with bootstrap support values using MEGA X (Kumar et al, 2018) to illustrate the relationship between amplified 16S rDNA gene of bacteria from test samples and other bacterial species.

RESULTS

Prevalence of Pathogens in Fish Feed

Results from the study indicated low to high prevalence of bacterial and fungal pathogens in fish-feed from all the farms. From the general screening of a total of 30

feed samples, 90% were positive for bacterial contamination and 70 % for fungal contaminations, with 40 % showing multiple contaminations with both bacterial and fungal pathogens (Table 2A). For the three species specific targets, *Streptococcus iniae, Streptococcus agalactiae and Staphyloccocus aureus* occurred at prevalence rates of 89 %, 60 % and 60% respectively, with *S. iniae* recording the highest. (Table 2B). Figures two and three are gel representations of amplified PCR products at their expected fragment sizes, confirming a profile of different species of bacterial and fungal contamination of fish-feed.

Table 2A. Prevalence - Prevalence Rate of Bacterial and Fungal Pathogens Based on Universal Bacteria and Fungi Primers in Fish Feed from Aquaculture Farms in Ghana

	Universal Bacteria	Universal Fungi	Co-contamination (UBF)
No of Positive Isolates	27	21	12
Prevalence Rate (Feed)	90 %	70 %	40 %

(UBF- Universal bacteria and fungi)

Table 2B: Prevalence Rate of Selected Bacteria Species in Fish Feed from Aquaculture Farms in Ghana

Target Bacteria Species	Prevalence (%) of Specific bacteria in samples		
	Tilapia	Feed	Water
Streptoccocus agalactiae	72%	60%	24 %
Streptoccocus iniae	88%	89%	32 %
Staphyloccocus aureus	14%	60%	0

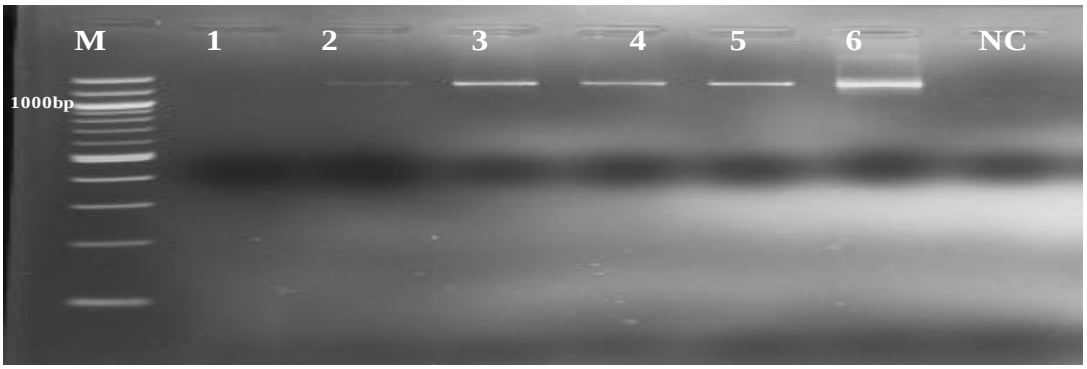

Figure 2: PCR Amplification of Bacteria Positive Fish Feed Samples at 1100bp with universal bacteria primer (M is Molecular marker, well 1 represents negative bacteria specimen; wells 2,3,4,5 and 6 were positive bacteria specimens whiles NC is negative control).

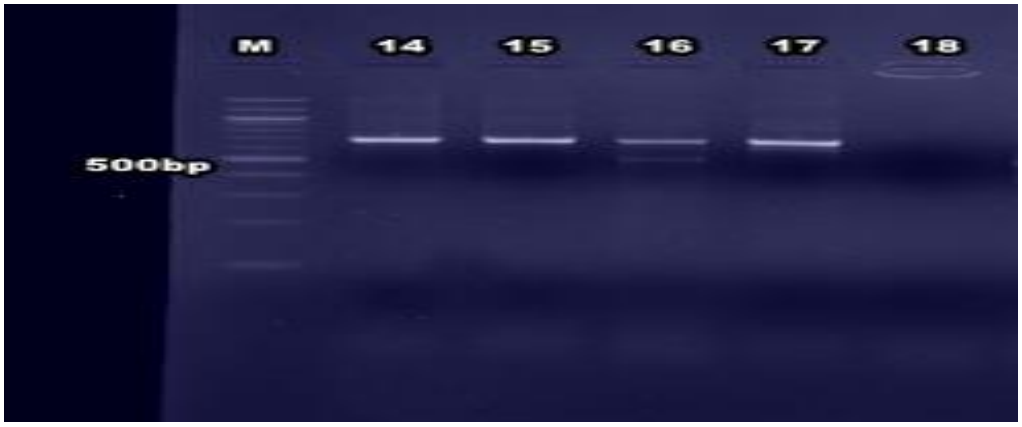

Figure 3: PCR Amplification of Fungi positive Fish Feed Samples (540bp) using universal fungi primer. (M is Molecular marker, wells 14, 15, 16 and 17 are fungi infested positive samples and 18 represents negative control).

Prevalence of Pathogens in Fish and Water Samples

Aside the general screening of samples for total bacteria and fungi in fish and water samples, all the three species (*Streptococcus agalactiae, Streptococcus iniae* and *Staphyloccocus aureus)* targeted for isolation for their significance in fish diseases in aquaculture farms especially, in tropical environments occurred at varying frequency rates in the various tissues of diseased and healthy fish samples (Fig. 5). High prevalence rates were recorded in external tissues than internal tissues (kidney, liver) (Fig 4). Prevalence of pathogens in gut contents (26.09%) occurred at similar rates to that recorded from diseased portions (26.89%) with no significant difference ($p>0.05$). *S. agalactiae* and *S. iniae* occurred at a prevalence rate of 24% and 32% respectively in the aquaculture water samples, however *Staphylococcus aureus* was not isolated from water samples (Table 2B). Generally, pathogens occurred at high prevalence rates in diseased fish (62.33%) than that in healthy fish (52.67%), however this difference was not significant ($P=0.14$) (Fig 5). There was also a positive correlation ($R=0.667$) between prevalence rate of pathogens in the fish feed and diseased fish (tilapia) which was significant ($P<0.05$).

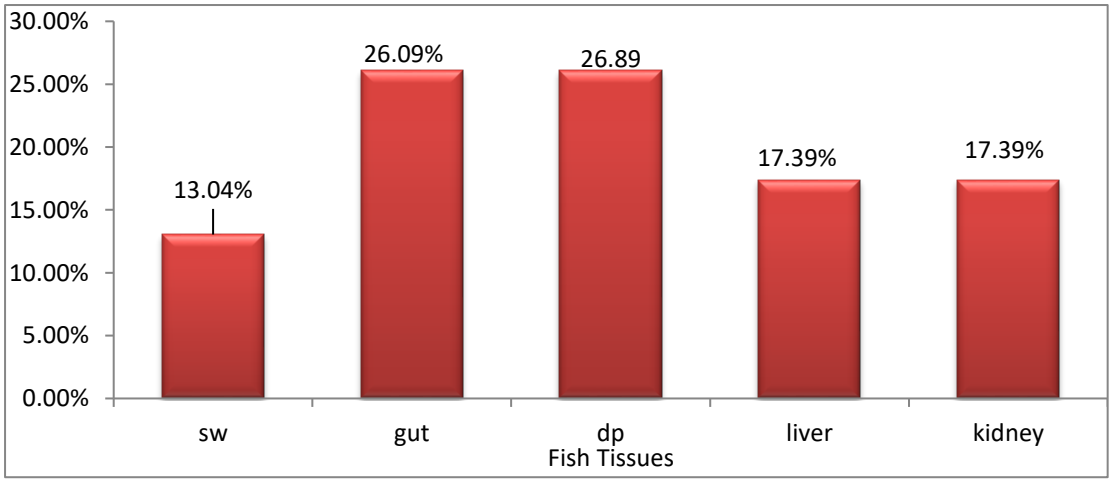

Figure 4: Prevalence of bacterial infections among various tissues and portions of fish analyzed (dp- disease portion of fish, sw, skin wash)

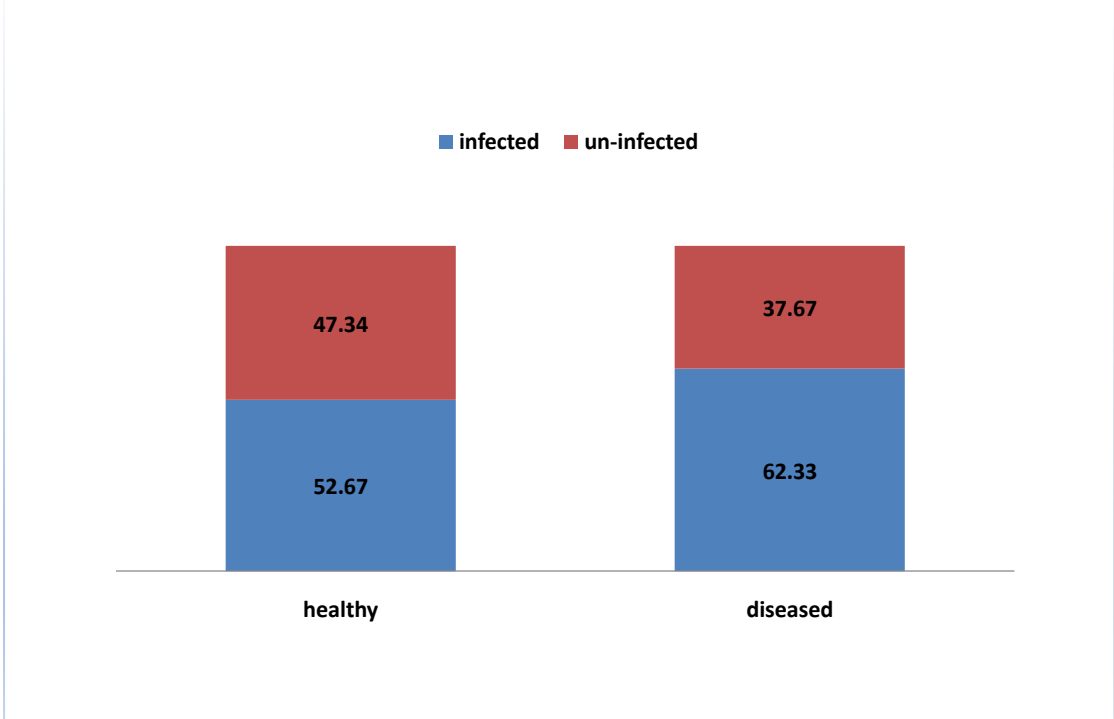

Figure 5: Prevalence rate (%) of bacterial infections per the health status of fish (p=0.14)

Quantification of Bacterial species Isolated in Fish-feed using qPCR

The quantity of pathogens is inversely proportional to the Ct values. For the species-specific quantifications, *S. agalactiae* recorded low concentrations with high Ct values between 39.60 and 41.90 and an average of 40.78 in the feed samples (Fig. 6b). However, for *S. iniae*, their concentrations per their Ct values were high (Ct:22-32) (Fig. 6a), consistent to their equally high frequency of occurrence in feed samples, which were significantly high ($P< 0.05$). *S. aureus* also recorded low to moderate concentrations in the fish feed samples, between 30.07 and 41.00 (Fig. 6c) with an average of 34.26 (Table 3).

Table 3 Threshold Frequency (Ct) values for the specific bacteria species amplified in fish-feed samples.

Sample ID	*S. agalactiae*	*S. iniae*	*S. aureus*
1	-	28.48	34.40
2	-	22.91	31.80
3	39.60	30.81	33.90
4	40.84	29.90	41.00
5	41.90	28.91	35.02
6	-	27.54	40.68
7	-	27.39	32.49
8	-	25.12	30.07
9	-	31.77	30.31
10	-	28.10	32.90
Average	**40.78**	**28.39**	**34.26**

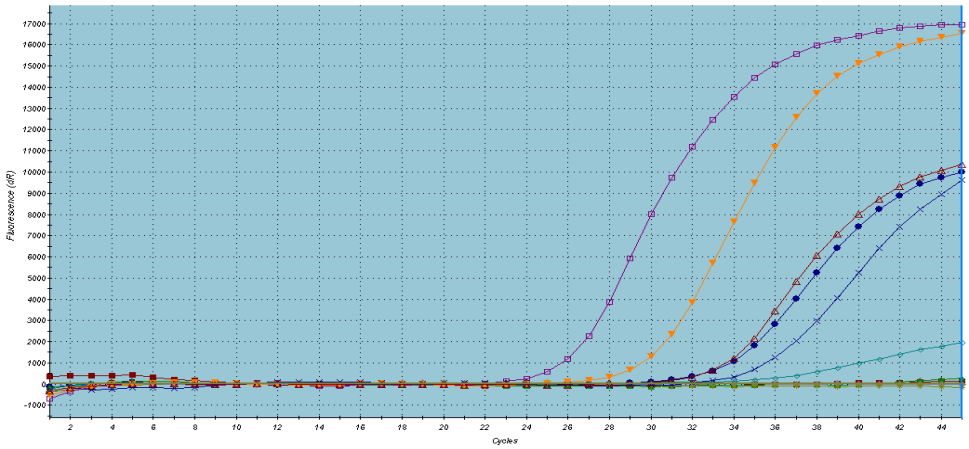

Figure 6(a) Amplification curve for *streptococcus iniae* in fish-feed samples

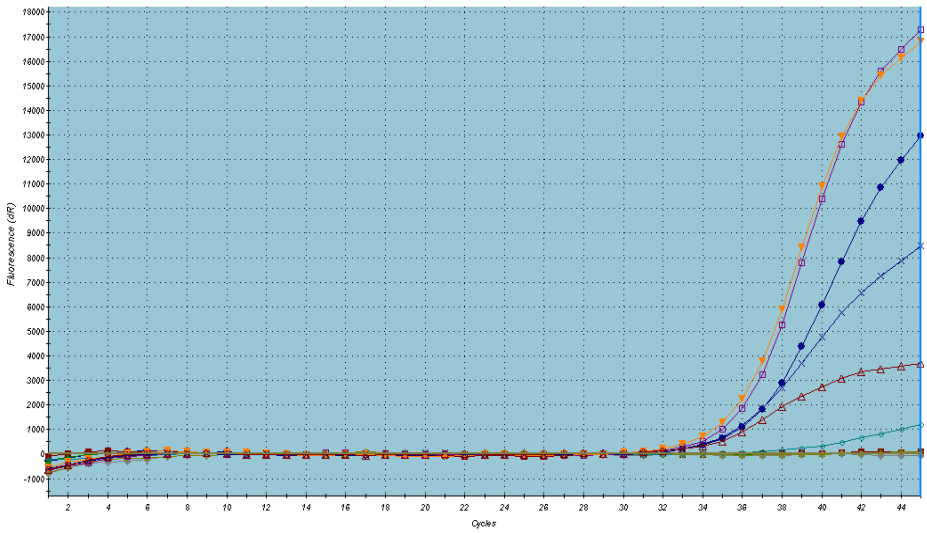

Figure 6(b) Amplification curve for *Streptococcus agalactiae* in fish-feed samples

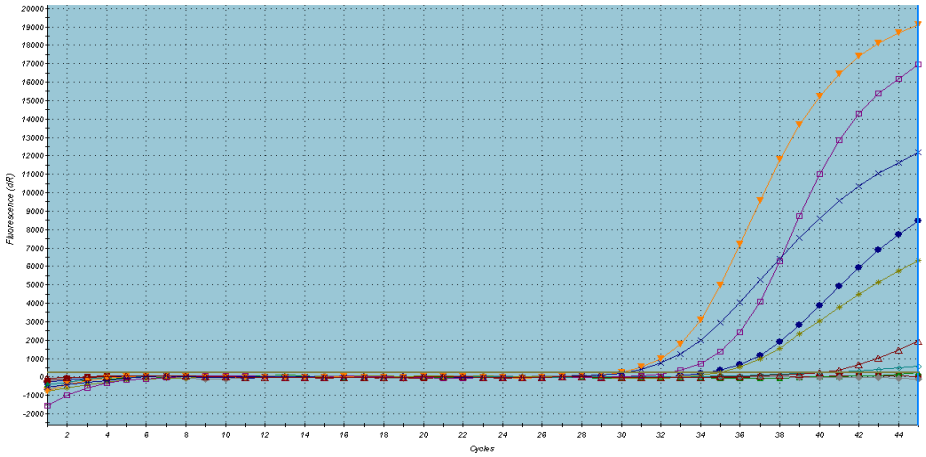

Figure 6(c) Amplification curve for *Staphylococcus aureus* in fish-feed samples

Sequence Analysis

Sequencing of universal bacteria using UG63f/r primer and isolated from fish feed confirmed the presence of targeted species and further revealed the presence of other non-target bacteria species. For instance, the blasting results revealed that different strains of *S. aureus* were isolated from the fish feed and had a very high genetic identity (99%) to strains from different sources; human, environment and livestock (Table 4). Thus, the variability of bacterial pathogens isolated from the feed was also evident with the sequence analysis of the bacteria gene that was undertaken. At 96 %, about three different genus of bacteria, including uncultured Caulobacter sp. clone CA8_41 16S ribosomal RNA gene, partial sequence (MF378493.1); Caulobacter leidyia strain MQS18 16S ribosomal RNA gene, partial sequence (GU046511.1); Sphingomonas sp. strain F25 16S ribosomal RNA gene, partial sequence, (MT756125.1); *Sphingomonas leidyi*; Alpha proteobacterium PI_GH4.1.G4 small subunit ribosomal RNA gene, partial sequence (AY162053.1), representing over 50 strains of each species, aligned with test samples in the fish feed. However, the Alpha proteobacterium and Caulobacter genus are all said to belong to Sphingomonas genus, thus a phylogeny of sequences downloaded from the NCBI website, revealed that test sequences from fish feed was identical to over six different *Sphingomonas species* (limited to less than 99 % identity) from various sources including; mineral water, water reservoir, hospital reservoir, contaminated soil and drinking water. Although all these sources were highly identical to sequenced data from this study, a much closer genetic identity was observed for the species sources from the hospital and water reservoir (Fig. 7)

Table 4: Description of *S. aureus* sequences isolated from fish feed on basic local alignment sequence tool (BLAST)

Identity (%)	Description	Accession number	source
99%	*S. aureus* strain NR_036903.1	MW453037	Human (urine)
99%	*S aureus* strain AN-SD10	MW362234.1	Environment (cosmetic face sponge)
99%	*S aureus* strain KHS103	CP063801.1	Livestock
Genus	uncultured Caulobacter sp. clone	MF378493.1	
Species	Caulobacter leidyia	MQS18	
Genus	Sphingomonas sp. strain F25 16S	MT756125.1	
Spcies	*Sphingomonas leidyi*	PI_GH4.1.G4	
Genus	Alpha proteobacterium	AY162053.1	

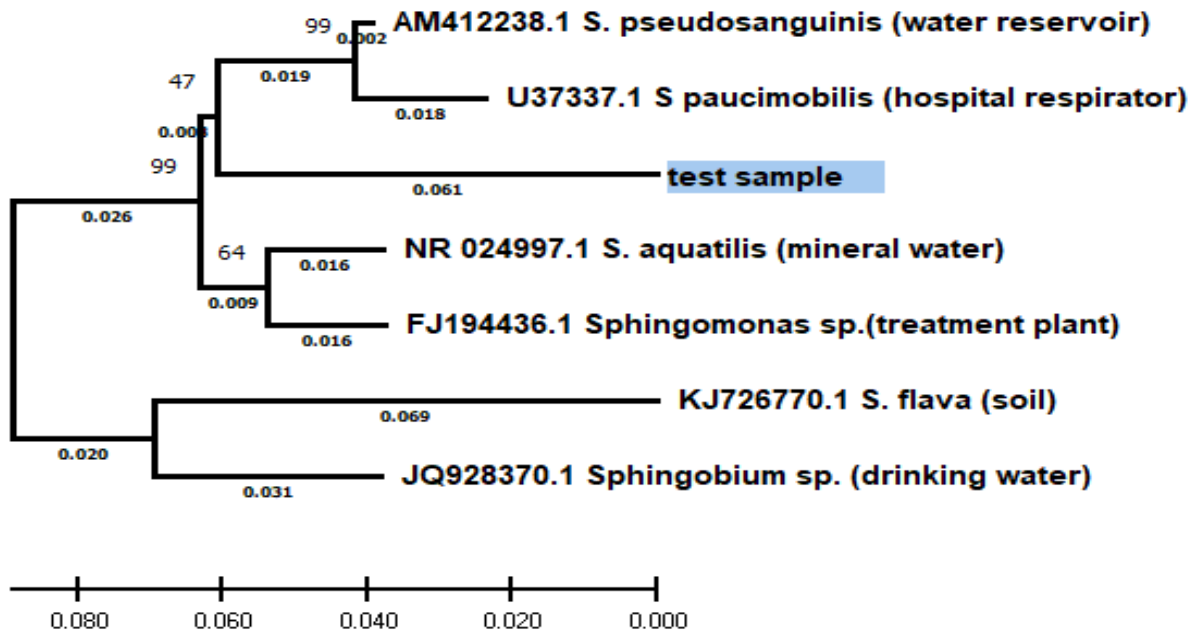

Figure 7. Genetic Comparison of Sequenced data (Sphingomonas sp) isolated from Fish Feed

(The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model (Tamura, 1992). The tree with the highest log likelihood (-3523.86) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. This analysis involved 7 nucleotide sequences. There were a total of 1480 positions in the final dataset. Evolutionary analyses were conducted in MEGA X)

DISCUSSION

This work reports the microbial profile of fish feed samples obtained from various aquaculture farms in Ghana from the amplification of bacterial and fungal genes using universal primers. Quite high frequencies and concentrations of the three targeted bacteria pathogens, were isolated from the feed, although some tested negative or below detection limit. At 96 % identity, three different genus of bacteria, and six different species (at 99 % identity), representing over 50 different strains were also identified from sequence analysis of fish feed. Generally, the bacteria load was higher than fungal load in the fish feed.

Among the targeted bacterial species, the quantification of the bacterial load increased from *S. agalactiae, S. aureus* to *S. iniae* in average Ct values of 40.78, 34.26 and 28.39 respectively, implying a concentration pattern of S. iniae >, S. aureus > S. agalactiae respectively. Recently, infections caused by *Streptococcus* spp. specifically, *S. agalactiae* and *S. iniae*, are the most predominant, causing huge economic losses to the tilapia industry in Ghana (Diyie et al.2022, Verner Jeffreys, et al. 2017). Multiple environmental factors, including warm water temperatures, increased ammonia levels, and low dissolved oxygen levels (caused by poor husbandry and high stocking density) (Bromage & Owens 2002) are presently considered the main contributing factors to their prevalence. However, findings from this work revealed that, all three bacteria species found in the feed samples were also present in the diseased fish samples at comparable prevalence rates, with no significant difference (Table 2B). Particularly, prevalence rate of

Streptococcus species *(S. iniae)* in feed were significantly higher (Table 2B) than that recorded from the background culture water, suggesting contamination of fish feed with this species, a contributing factor to streptococcosis in fish. Again, among the tissues analyzed, there was high prevalence and quantities (Table 3) of bacteria isolated from the gut content of the diseased fish, similar to that from disease portions with no significant difference ($P>0.05$) (Fig 4). This study also recorded a strong positive correlation of 0.667 between the prevalence of bacteria in the fish feed and that in the tilapia. This indicates that an increment in bacteria prevalence or load in the fish feed would lead to an increment in bacteria prevalence or load in the tilapia and vice-versa. All these analyses go to establish that, microbial contamination of fish feed is an important source of entry for pathogens into the aquaculture system. Results again corroborates the findings by Su et al., (2016), who also developed quantitative PCR to monitor *S. agalactiae* colonization on mucosal surfaces and tissue tropism, and found high bacteria loads in the intestines than in the muscles. Contrary to expectations, *Staphylococcus aureus* was the only species not isolated from the culture water samples collected, however it occurred at a significantly higher prevalence rate ($p < 0.05$) in the fish feed samples as well as in diseased fish (Table 2B). According to Singh & Kulsherestha, (1993), Staphylococci in fish and fish products has been traced to handlers and fish farmers. Thus, contamination of fish feed with *S. aureus* as recorded in this study, represents a direct source of this pathogen in fish through human handling of feed either during preparation or feeding. Also evident from the sequence analysis, the strain of *S. aureus* isolated from the fish feed in this study had a very high similarity with strains that are present in humans as well as the environment (Table 4), and since fish farmers and other handlers are always in direct contact with the fish, a direct route of transmission is inevitable. Actually, *S. aureus* related disease has over the last ten years cause significant global problem with economic hardships on both aquaculture and veterinary as a whole, causing additional food safety concerns (Aires-de-Sousa, 2017). Thus, identification of potential sources for possible elimination can help reduce staphylococcus infections.

For the non-tarted species identified via sequenced analysis, the cycling threshold (Ct) values of the universal bacterial, recorded significantly lower Ct values ($P<0.05$), consequently high DNA than those of the specific species targeted. This was an indication of the presence of more other bacteria species in the feed than those isolated. This was therefore confirmed by the sequence analysis which revealed the presence of over 50 different bacteria species and strains belonging to the genera commonly referred to as sphingomonads. The strain, *Sphingomonas leidyi*, strain IIL-Asp35, 16S ribosomal RNA gene, partial sequence with accession number KX380920.1 at 96 % identity in the feed samples, was also found in the liver of the fish at 98 % identity. This represents evidence that, the presence of these species in the feed, eventually find their way into the internal organs of fish, through feeding. The genus sphingomonads accommodate the alpha-proteobacterium species that contain glycosphingolipids (GSL) in its cell envelope. These species are known to be widely distributed and have been isolated from different water and land habitats, particularly from environments contaminated with toxic compounds, where they use the contaminant as nutrients (Balkwill et al., 2010). Sphingomonadaceae initiate biofilm membrane formation that is used in full scale operation and laboratory simulation experiments (Glaeser *et al*., 2014). Also, sphingomonads are often used in the production of extracellular polymers like sphingans (e.g., gellan, welan) used extensively in the food industries

(Yabuuchi & Kosako, 2015) due to their biodegradative and biosynthetic capabilities and could be the source of introduction of this species into the commercial feeds. Although generally not a human pathogen(Chandra & Kronenberg, 2015), the species *Sphingomonas paucimobilis* are said to be occasional human pathogens, thus zoonotic potential has been hypothesized in some species (Steinberg, & Burd, 2015).

Based on questionnaire analysis, observations and interactions on farms, feeds were mainly packed and stacked together in barns and store houses for quite a long time. This storage system could enhance the thriving of pathogens, especially molds and their subsequent transfer to the fish through feed. From this study, feed contaminated with fungi occurred at a prevalence rate as high as 70% and a potential source of fungal infections in fish. During sampling, fish showing clinical signs of fungal and bacterial diseases varying from reddish patches, ulcerative lesions, exophthalmia to septicemia were all recorded from each of the farms sampled. Also, from the questionnaire administered, farmers admitted to the appearance of lesions on fish, days after being fed with feed that has been kept for months. Similar observations were made in a study by Solomon et al., (2016), where fish feed stored up to six months, and opened to atmospheric conditions recorded changes in nutrient quality with growth of molds and appearance of lesions in fish subsequently fed by it.

Although, not fully disclosed by all farms, two farms admitted the inclusion of some additives, one wheat and the other trash fish and other farm-made preparations including extraction of oils from fish intestines to the feed. These additives usually keep the feed moist, facilitate fungal and bacteria growth in feed and tend to pose a higher risk of contaminating and transmitting diseases than commercial feeds. Budiati et al. (2015), also indicated th addition of chicken offals and spoiled eggs to feed as a potential source for the bacterial contamination to water and fish, with evidence highlighting the importance of feed quality in aquaculture system. On the other hand, the pathogen load in commercial feeds as recorded in this study is also an indication that there can be equal contamination of feed with pathogens regardless the source under prolonged and favorable environmental conditions. Thus, prevalence rate of pathogens as recorded in this study, could be attributed to the mode and duration of feed storage, addition of other ingredients as well as handling during feeding**.**

Ingestion of these bacteria in contaminated feed at the quantities recorded in this study may not be significant enough in causing disease in single feed ration. However, the large input of these nutritionally quality feed contaminated with microbes administered over a prolonged period of time could result in accumulation of these pathogen and significantly cause major disease outbreaks, through the direct uptake from the feed and/or contamination of water, for subsequent infestation of fish as observed in some farms. Thus, this important component of aquaculture production, covering over 50 to 60 percent of production cost could be the source of the observable clinical signs of fish diseases, a major constraint in aquaculture production.

Feed is at the beginning of the food safety chain, administered daily and directly into the water, hence, represents a significant potential pathway for entry of pathogens and chemical contaminants into the human food supply. Thus, ensuring that fish-feed is free of bacterial pathogens should help reduce infections in fish and also in humans as pathogens can be transmitted through the food chain to humans and cause human foodborne illness. Recorded from the present study, bacteria species recovered from fish-feed and diseased fish also

occurred at similar frequencies and quantities in healthy fish. This is an indication that these organisms may sometimes not constitute a serious threat to the fish up to the point of marketing, due to their inherent resistance, nonetheless, the same pathogen load could be of public health significance. In this regard, proper treatment of fresh fish is a necessity for human safety. Fish and fish products have been reported as means of food-borne bacterial infections in humans (Novotyn et al. 2004, Hastein et al. 2006, Efuntoye et al. 2012). Rather than trying to treat every disease case, it is advisable to follow a preventive approach before the event of any disease outbreaks.

CONCLUSION

The prevalence of bacterial and fungal contamination of fish feed in quantities significantly comparable to that from the gut contents of diseased fish has been confirmed through this study. Also, a positive correlation was found to exist between prevalence rate of pathogens in fish feed and diseased fish, thus, implicating fish feed as a significant source of disease pathogens unto fish farms. Advancements towards the safety of fish should therefore include the surveillance of fish-feed for microbial quality, and incorporation into human foodborne disease surveillance systems to ensure holistic effectiveness.

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COMPETING INTERESTS

The authors declare no competing interest.

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