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RESEARCH PAPER

Assessment of coliforms and bacterial loads associated with skin, gills, intestines, and muscles of five species of grouper

Ahmed H. Al-Harbi [1]*, Abdulmohsen I. Al-Asous [1]

[1]*Sustainability and Environment Sector, King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia*

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Corresponding Author

Ahmed H. Al-Harbi

E-mail: aalharbi1377@gmail.com
Tel: + 966542191377

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Abstract

This study was conducted to determine the total coliform (TC) and fecal coliform (FC) counts and bacterial loads of the skin, gills, intestines, and muscles of grouper, ariolated grouper (*Epinephelus areolatus*), orange-spotted grouper (*Epinephelus coioides*), squaretail coralgrouper (*Plectropomus areolatus*), roving coral grouper (*Plectropomus pessuliferus*), and yellow-edged lyretail grouper (*Variola louti*). The aerobic bacterial counts of the groupers' skin, gill, intestine, and muscle samples ranged from 9.04 ± 0.14 to 11.57 ± 0.82, 7.69 ± 0.89 to 11.51 ± 0.51, 7.49 ± 0.14 to 11.54 ± 0.41, and 4.41 ± 0.49 to 4.64 ± 0.35 log CFU/g, respectively. The total coliform counts were presented in the skin with values of 1100 MPN/g, while in the gills and intestines, the values ranged from 460 to 1100 MPN/g and 240 to 460 MPN/g, respectively. The fecal coliform counts were found in skin and gill samples, with the values ranged from 150 to 240 MPN/g and 64 to 150 MPN/g, respectively. In contrast, the total coliform and fecal coliform counts were not detected in all the groupers' muscle samples.

Introduction

Groupers **(**Serranidae: Epinephelinae**)** are widely distributed across tropical and subtropical seas worldwide and are considered the most important commercial fish species (Craig et al., 2011). The ariolated grouper (*Epinephelus areolatus*), orange-spotted grouper (*Epinephelus coioides*), squaretail coralgrouper (*Plectropomus areolatus*), roving coral grouper (*Plectropomus pessuliferus*), and Yellow-edged lyretail grouper (*Variola louti*) are the most important grouper species in Saudi Arabia due to their high consumer demand and market value (Al-Harbi & Al-Asous, 2022).

Fish and seafood products are among the most highly perishable food products. The quality of fresh fish is a significant concern for both industry and consumers worldwide. The quality of seafood begins to deteriorate immediately after capture and continues during storage, resulting in approximately 10% of the global seafood harvest being spoiled annually (Svanevik & Lunestad, 2011).

The storage temperature, handling, and processing conditions of fish and seafood products lead to increased growth of spoilage and pathogenic microorganisms. Generally, the skin, gills, and digestive tract of fish contain diverse bacterial populations, whereas the muscle tissue of healthy individuals is considered to be free of bacteria. It is estimated that the bacterial populations normally range from 10^2 to ~10^4 bacteria cm^2, on the skin of fish, up to 10^6 bacteria g^{-1} in the gills, and dense bacterial populations in the digestive tract, up to 10^8 aerobic bacteria and 10^5 anaerobic bacteria g^{-1} (Austin, 2006).

Fish and seafood products can act as reservoirs for several bacterial pathogens associated with human diseases, including *Aeromonas* spp., *Campylobacter jejuni*, *Clostridium* spp., *Edwardsiella tarda*, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium* spp., *Salmonella* spp., *Staphylococcus aureus*, *Streptococcus iniae*, *Vibrio* spp., and *Yersinia* spp. (Heinitz & Johnson, 1998; Heinitz et al., 2000; Al-Harbi & Uddin, 2004; Novotny et al., 2004; Al-Harbi & Uddin, 2005; Pao et al., 2008; Novoslavskij et al., 2016; Sheng & Wang, 2021; Walayat et al., 2023).

Since the fish skin, gills, and intestinal microorganisms are the major causative agents of fish and seafood products spoilage (Al-Harbi & Uddin, 2005; Austin, 2006). Therefore, these bacteria quickly invade muscle blocks after being captured. It is well known that the enumeration of aerobic plate counts (APC) and the coliform group counts is used as an indicator of the freshness and quality of fish and seafood products. This study aimed to assess the aerobic bacterial load, as well as the total coliform (TC) and fecal coliform (FC) levels, in the skin, gills, intestines, and muscles of five commercially important grouper species in Saudi Arabia.

Materials and Methods

Sample collection

A total of 50 individual fish specimens representing five grouper species (10 fish/species) used in this study were collected from a local seafood market in Riyadh, Saudi Arabia (Table 1). The samples were wrapped individually in a sealed plastic bag and immediately transported to the laboratory within an hour in iced styrofoam boxes. Upon arrival at the laboratory, the total bacterial counts and coliform group counts were determined individually for each sample in the skin, gills, intestines, and muscles of the groupers. These species were selected because of their high commercial importance in Saudi markets (Al-Harbi & Al-Asous, 2022).

Table 1. Means of total length and weight of groupers samples

Common name	Scientific name	Length	Weight
Ariolated grouper	*Epinephelus areolatus*	38.0 cm	599.5g
Orange-spotted grouper	*Epinephelus coioides*	47.6 cm	1650.7g
Squaretail coral grouper	*Plectropomus areolatus*	52.0 cm	2118. 7g
Roving coral grouper	*Plectropomus pessuliferus*	52.9 cm	1777.2g
Yellow-edged lyretail grouper	*Variola louti*	53.3 cm	1772.1g

Sample preparation

Fish skin

Samples from different locations of the fish skin were gently taken by rubbing a sterile cotton swab several times over the skin from both sides of each fish (from the operculum to the caudal peduncle) and then inoculated into 2 ml of sterile physiological saline solution (0.85% NaCl), and serial decimal dilutions of the homogenates were prepared up to 10^{-7}.

Fish muscle

The external surface of the fish was wiped with 70% ethanol, and the muscle sample was taken from the flesh of the anterior dorsal area between the head and the dorsal fin. Approximately 10 g of the underlying muscle flesh was sampled from the

bone of the fish muscle using sterilized scalpels and forceps, mixed with 90 ml of sterile physiological saline solution (0.85% NaCl), and homogenized separately in a sterilized Waring Blender (IUL Instruments, Spain) for 1 min. A series of 10-fold dilutions of the homogenates was prepared up to 10^{-7}.

Fish gill

The external surface of the fish was wiped with 70% ethanol, and the gill of each fish was removed from both sides of the filament and homogenized separately in a mortar aseptically. Around 2 g of wet homogenate was inoculated into 18 ml of sterile physiological saline solution (0.85% NaCl), and serial decimal dilutions of the homogenate were prepared up to 10^{-7}.

Fish intestine

The external surface of the fish was wiped with 70% ethanol, and the intestines of each fish were removed and homogenized separately in a mortar aseptically. Around 1 g of wet homogenate was inoculated into 9 ml of sterile physiological saline solution (0.85% NaCl), and serial decimal dilutions of the homogenate were prepared up to 10^{-7}.

Microbiological analyses

The aerobic plate count (APC as CFU per unit of sample), total coliform (TC), and fecal coliform (FC) counts of the skin, gill, intestine, and muscle from each fish were determined.

Aerobic plate counts (APC)

To determine the aerobic plate counts (APCs) of the skin, gills, intestines, and muscles of the grouper, 0.1 ml of each dilution (10^{-1} – 10^{-7}) was spread on the surface of tryptone soya agar (TSA; Oxoid) plates in duplicate and incubated at 30ºC for 48 h. The colony-forming units (CFU) were counted with a Quebec Darkfield Colony Counter (Leica, Inc., Buffalo, New York). The plates having ≥ 30 to 300 colonies were used to calculate bacterial population numbers, expressed as CFU. All analyses were performed in triplicate.

Coliforms

The total coliform (TC) and fecal coliform (FC) counts of the skin, gills, intestines, and muscles of grouper samples were determined using the three-tube Most Probable Number (MPN) (APHA 9: 2015) methods (Kornacki et al., 2015). Briefly, 1 ml of each dilution (10^{-1} – 10^{-3}) of the skin, gill, intestine, and muscle of the grouper was transferred to lauryl sulphate broth (Lauryl Tryptose Broth) tube and incubated at 35ºC for 24 - 48 h. The formation of gas within 24 - 48 h was considered as evidence for presumptive coliforms. A loopful of suspension from each positive LST tube was transferred into a brilliant green lactose bile broth (BGLBB) tube and incubated at 35ºC for 24 – 48 h, and the formation of gas within 24 - 48 h was considered as evidence for confirmed coliforms. A loopful of suspension from each positive BGLBB tube was transferred into an *Escherichia coli* (EC) broth tube and incubated at 44.5 ºC for 24 – 48 h, and the formation of gas within 24 - 48 h was considered as evidence for fecal coliform counts. The MPN index and values for total coliform and fecal coliform counts were calculated from the number of positive BGLBB tubes and EC broth tubes, respectively.

Statistical analysis

All experiments were performed in triplicate, using skin, gills, intestines, and muscles from five species of grouper, and the data are expressed as mean ± standard deviation. The significance of the difference between the sample mean values was compared using a one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p \leq 0.05$.

Results and Discussion

Bacterial load

The bacterial loads in the skin, gills, intestines, and muscle samples of ariolated grouper, orange-spotted grouper, squaretail coralgrouper, roving coral grouper, and yellow-edged lyretail grouper are presented in Tables 2-6. The aerobic bacterial counts in the groupers' skin, gill, and intestine samples ranged from 9.04 ± 0.14 to 11.57 ± 0.82, 7.69 ± 0.89 to 11.51 ± 0.51, and 7.49 ± 0.14 to 11.54 ± 0.41 log CFU/g, respectively, whereas the CFU g^{-1} of the groupers' muscle samples ranged from 4.41 ± 0.49 to 4.60 ± 0.29. In the present study, the aerobic bacterial counts in the skin, gills, and intestines of all five grouper species were markedly higher than those reported by Austin (2006). Higher total aerobic bacterial counts could have resulted from contamination

during transportation, handling, processing, storage, and/or elevated temperatures during transportation and processing (Al-Harbi & Al-Asous, 2022).

Although fish muscles are generally considered sterile, the muscle samples of all five species of grouper used in this study were comparatively contaminated by bacteria, but still within acceptable limits, below $5x10^5$ CFU/g^{-1} set by the ICMSF standard (1986) as a measure of good quality food product. The differences in aerobic bacterial counts in muscle samples among all five grouper species were not significant ($p > 0.05$). Several other studies have reported the detection of bacteria in grouper muscles, including brown spotted grouper (*Epinephelus chlorostigma*), stored in dry ice with values of (10^6 CFU/g) (Jeyasekaran et al., 2008), wire-netting reef cod (*E. merra*), stored in melting ice with values of (10^5 CFU/g) (Jeyasekaran et al., 2005), white grouper (*E. aeneus*), stored in ice and at chill temperature (4 °C) with values of (10^3 CFU/g) (Özogul et al., 2008), orange-spotted grouper (*E. coioides*), stored in ice with values of (10^2 CFU/g) (Sharifian et al., 2014) and 2.9048 log CFU/g, in fillets of yellow grouper (*E. awoara*) stored under vacuum packaging at 0°C, (Li et al., 2011).

In addition, bacterial populations were observed in the muscles of different fish species, such as African catfish (*Clarias gariepinus*) (El-Gendy et al., 2024), Atlantic horse mackerel (*Trachurus trachurus*) (Alfaro et al., 2013), Atlantic mackerel (*Scomber scombrus*) (Albertos et al., 2017; De Alba et al., 2019), haddock (*Melanogrammus aeglefinus*) (Karim et al., 2011), herring (*Clupea harengus*) (Karim et al., 2011; Albertos et al., 2019), Nile tilapia (*Orechromis niloticus*) (Mandal et al., 2009; Onjong et al., 2018), sardine (*Sardina pilchardus*) (Nuñez-Flores et al., 2013), sea bass (*Dicentrarchus labrax*) (Papadopoulos et al., 2003), and sutchi / pangasius catfish (*Pangasianodon hypophthalmus*) (Viji et al., 2015; El-Gendy et al., 2024).

In the present study, the bacterial load in muscle samples was significantly lower than that in the skin, gills, and intestines ($p < 0.05$) (Tables 2-6 and Figure 1).

These findings are consistent with earlier reports on other subtropical marine fish species, including silver trevally *Pseudocaranx dentex*, snapper *Pagrus auratus*, and sea mullet *Mugil cephalus* (Al Bulushi et al., 2008). Mandal et al., (2009) reported that the muscle samples of Nile tilapia (*Orechromis niloticus*) had lower bacterial loads than the gills and intestines.

The differences in the aerobic bacterial load levels in the skin, gills, intestines, and muscles among all five species of grouper were primarily due to local fish market practices, including handling, processing, and storage.

Coliforms

The coliform group counts in the skin, gills, intestines, and muscles of the five grouper species are presented in Tables 2-6 and Figures 2 and 3. The total coliform (TC) counts were presented in skin, with a value of 1100 MPN/g, while the values in gills and intestines ranged from 460 to 1100 MPN/g and from 240 to 460 MPN/g, respectively. Fecal coliform (FC) counts were found in the skin and gills, with values ranging from 150 to 240 MPN/g and from 64 to 150 MPN/g, respectively. In contrast, the total coliform (TC) and fecal coliform (FC) counts were not detected in the muscles of any of the five species of groupers (Tables 2-6 and Figures 2 and 3). Furthermore, fecal coliform (FC) counts were not detected in the intestines of any of the five species of groupers (Tables 2-6 and Figure 3). However, several previous studies have reported the detection of coliform group bacteria in grouper muscles, including wire-netting reef cod (*Epinephelus merra*) (Jeyasekaran et al., 2005), brown spotted grouper (*E. chlorostigma*) (Jeyasekaran et al., 2008), and wild white grouper (*E. aeneus*) (Özogul et al., 2008). Moreover, coliform group bacteria also have been reported in muscles of several other fish species, such as African catfish (*Clarias gariepinus*) (El-Gendy et al., 2024), Atlantic mackerel (*Scomber scombrus*) (Albertos et al., 2017; De Alba et al., 2019), channel catfish (*Ictalurus punctatus*) [(Marshall & Jindal, 1997), haddock (*Melanogrammus aeglefinus*) (Karim et al., 2011), herring (*Clupea harengus*) (Karim et al., 2011; Albertos et al., 2019), tilapia (*Orechromis* spp.) (Pao et al., 2008; Mandal et al., 2009; Gatti-Junior et al., 2014; Onjong et al., 2018; Thaotumpitak et al., 2022), sardine (*Sardina pilchardus*), sea bass

(*Dicentrarchus labrax*) (Papadopoulos et al., 2003), and sutchi/pangasius catfish (*Pangasianodon hypophthalmus*) (Viji et al., 2015; El-Gendy et al., 2024).

Table 2. Mean values of total bacterial counts and coliforms group count of the skin, gill, intestine and muscle of ariolated grouper (*Epinephelus areolatus*) stored in ice

Sample type	Bacterial counts	Total coliforms	Fecal coliforms
Skin	11.6 ± 0.82	1100	150
Gill	11.5 ± 0.51	1100	150
Intestine	11.5 ± 0.41	460	ND
Muscle	4.5 ± 0.41	ND	ND

Mean ± SD standard deviations; ND: Not detected

Table 3. Mean values of total bacterial counts and coliforms group count of the skin, gill, intestine and muscle of orange-spotted grouper (*Epinephelus coioides*) stored in ice

Sample type	Bacterial counts	Total coliforms	Fecal coliforms
Skin	9.5 ± 0.71	1100	240
Gill	8.4 ± 0.14	1100	93
Intestine	7.7 ± 0.89	240	ND
Muscle	4.6 ± 0.29	ND	ND

Mean ± SD standard deviations; ND: Not detected

Table 4. Mean values of total bacterial counts and coliforms group count of the skin, gill, intestine and muscle of squaretail coralgrouper (*Plectropomus areolatus*) stored in ice

Sample type	Bacterial counts	Total coliforms	Fecal coliforms
Skin	10.6 ± 0.32	1100	240
Gill	9.1 ± 0.39	1100	150
Intestine	7.5 ± 0.14	240	ND
Muscle	4.4 ± 0.49	ND	ND

Mean ± SD standard deviations; ND: Not detected

Table 5. Mean values of total bacterial counts and coliforms group count of the skin, gill, intestine and muscle of roving coral grouper (*Plectropomus pessuliferus*) stored in ice

Sample type	Bacterial counts	Total coliforms	Fecal coliforms
Skin	9.0 ± 0.14	1100	150
Gill	9.0 ± 0.14	460	64
Intestine	8.6 ± 0.82	460	ND
Muscle	4.6 ± 0.11	ND	ND

Mean ± SD standard deviations; ND: Not detected

Table 6. Mean values of total bacterial counts and coliforms group count of the skin, gill, intestine and muscle of Yellow-edged lyretail grouper (*Variola louti*) stored in ice

Sample type	Bacterial counts	Total coliforms	Fecal coliforms
Skin	9.9 ± 0.92	1100	240
Gill	8.8 ± 0.49	1100	93
Intestine	8.3 ± 0.53	240	ND
Muscle	4.4 ± 0.14	ND	ND

Mean ± SD standard deviations; ND: Not detected

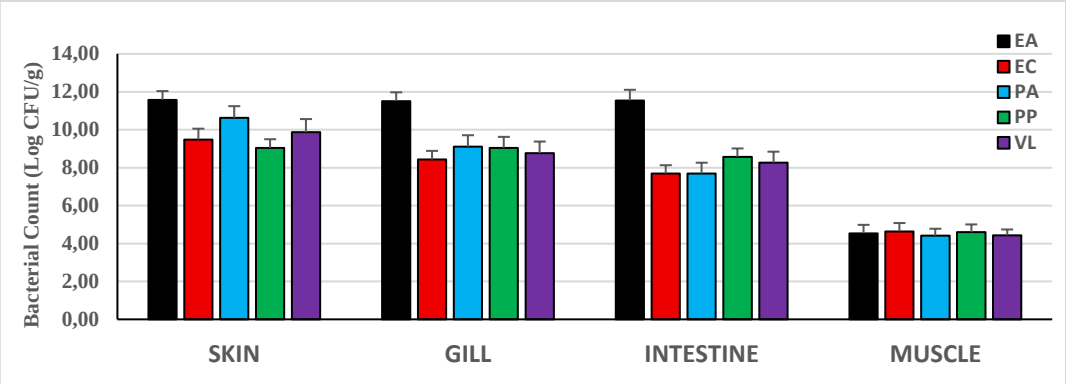

Figure 1. Mean values of bacterial count (log CFU/g) of the skin, gill, intestine, and muscle of five species of grouper. EA= Ariolated grouper, EC= Orange-spotted grouper, PA= Squaretail coralgrouper, PP= Roving coral grouper, and VL= Yellow-edged lyretail grouper.

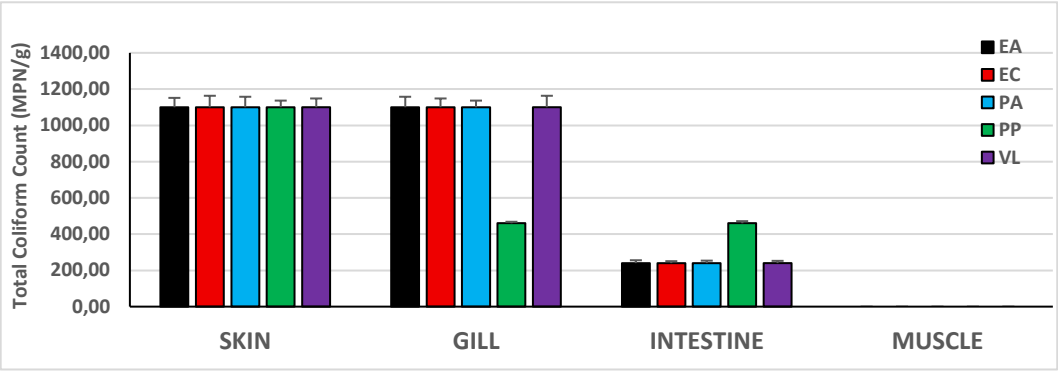

Figure 2. Mean values of total coliform count (TC) of the skin, gill, intestine, and muscle of five species of grouper. EA= Ariolated grouper, EC= Orange-spotted grouper, PA= Squaretail coralgrouper, PP= Roving coral grouper, and VL= Yellow-edged lyretail grouper.

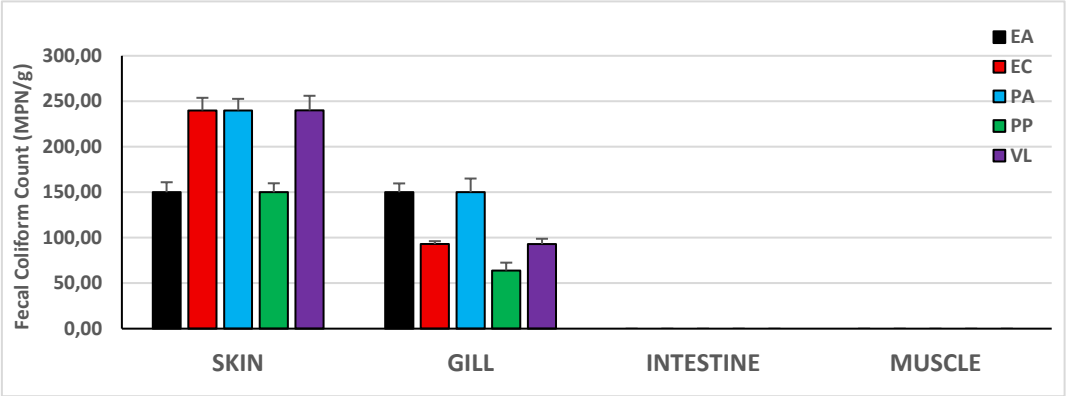

Figure 3. Mean values of fecal coliform count (FC) of the skin, gill, intestine, and muscle of five species of grouper. EA= Ariolated grouper, EC= Orange-spotted grouper, PA= Squaretail coralgrouper, PP= Roving coral grouper, and VL= Yellow-edged lyretail grouper.

In addition, Atwill and Jeamsripong (2021) determined the prevalence of fecal coliforms in seafood samples consisting of Pacific white shrimp, oysters, blood cockles, and Asian seabass to be equal to 100%, with a total average concentration (± SD) of 9×10^4 MPN/g (± 4×10^4 MPN/g). Moreover, the incidence of total coliforms and fecal coliforms has been reported in pond water, sediment, and the intestines of farmed hybrid tilapia (*Oreochromis niloticus Oreochromis aureus*) (Al-Harbi, 2003).

The prevalence of fecal coliform counts in *Epinephelus coioides*, *Plectropomus areolatus*, and *Variola louti* skin was higher than that observed for *Epinephelus areolatus* and *Plectropomus pessuliferus* skin, while higher fecal coliform counts were observed in *Epinephelus* areolatus and *Plectropomus*

areolatus gills than in *Epinephelus coioides*, *Plectropomus pessuliferus*, and *Variola louti* gills (Tables 2-6 and Figure 3). The detection of fecal coliforms in grouper skin and gill samples suggests lapses in hygiene during handling, transportation, processing, or storage. Furthermore, contaminated ice can act as a source of pathogenic bacteria, including fecal coliforms, and may contribute to the transmission of foodborne diseases (Falcão et al., 2002; Gerokomou et al., 2011; Economou et al., 2016).

Conclusions

In conclusion, this study successfully investigated coliform counts and bacterial loads associated with the skin, gills, intestines, and muscles of five species of grouper (*Epinephelus areolatus*, *Epinephelus coioides*, *Plectropomus areolatus*, *Plectropomus pessuliferus*, and *Variola louti*) stored in ice. The skin, gills, and intestines of the five grouper species had a higher bacterial load than that in the fish muscles. Overall, muscle tissue samples from all five grouper species were free of coliform contamination. Further investigation is required to identify the bacterial communities in the skin, gills, intestines, and muscles of the grouper.

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