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Bacterial load comparison of marine fish collected and commercially obtained for human consumption in western region of Yucatan Peninsula, Mexico

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Abstract: This study determined the presence of human pathogenic bacteria in fish muscle, recollected at moment of their capture and during their process for sale in marketing sites. The samples were recollected in eighth touristic and fishing zones of Mexican Caribbean. Samples were inoculated by duplicates in agar plates of specific environment of: S-S, EMB and TCBS and were incubated at 36ºC during 24 hrs.. Strains were identified according to Merck (1994) criteria and finally, in order to confirm the identification with the commercial kit API-20E. Species of different bacterial families were identified, such as: Enterobacteriaceae; *Salmonella hirschfeldii, Salmonella schottmulleri, Salmonella parathipi, Salmonella typhi, Salmonella enteritidis, Proteus miriabilis, Proteus rettgeri, Proteus vulgaris, Citrobacter freundii, Citrobacter amanolaticus* and three varieties of *Escherichia Coli*; Pseudomonadaceae: *Pseudomonas fluorescens;* Aeromonadaceae: *Aeromonas hydrophila* and Vibrionaceae: *Vibrio fluviales, V. cholerae El Tor* and *V. parahaemolyticus.* The 70.6% of the samples presented a bacterial growth; 46% in recently captured fish, and 54% in market fish. It was demonstrated that fish is already contaminated by pathogenic bacteria in their natural environment and that handling during their commercialization does not significantly increase their bacterial load.

Key words: Pathogenic bacteria, fish muscle, fecal contamination, public health.

Introduction

The main source of marine water contamination in the Mexican Caribbean is constituted by domestic wastewater mainly due to feces haulage, causing entry of pathogenic bacteria in coastal marine environment. Despite that this water go throw mechanisms of dilution and mix, these can be unable to self-purify the contamination, when their concentrations are above the ocean capacity (Botello *et al*., 2005).

In contaminated marine water intestinal pathogens are frequent, such as *Salmonella typhi* and *Salmonella paratyphi* A and B, responsible of typhoid and paratyphoid fever, and gastroenteritis. Infections caused by these bacterias can be transmitted not only by water consumption, but also by the intake of fish and shellfish from contaminated waters. Some authors register that salinity is the main factor for enteric bacteria viability. Bacterias that cause typhoid fever present a high survival capacity, even in adverse conditions and remain viable in seawater in a range of 2 to 12 weeks (Cabral, 2010).

Fish and other marine organisms that live in contaminated coastal water, poses a microbial microflora dependent on the existing in the waters where they live. In the mucus that covers the external surface of the fish, it has been identified bacteria of the genus: P*seudomonas, Salmonella, Micrococcus, Sarcina, Serratia* and *Vibrio* (Roberts, 1999). Regardless of the type of alimentation of the fish, which ingest bacteria on their food, it is registered a large number of these microorganisms in their digestive tract and epidermis, where also it has been identified species of genus *Pseudomonas, Escherichia, Salmonella, Streptococcus, Staphyloco-*

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ccus, *Clostridium* and *Vibrio* (Alam *et al*., 2006).

Bacterias found in skin and gastrointestinal content of the live fish, do not invade the muscular package because the organism is protected by its natural defenses. Also, pathogenic enterobacteria can get in touch with the fish as a consequence of handling by infected people or healthy carriers, during the capture, transport, elaboration and preparation process (Angeles, 2012).

Early studies about pathogenic bacteria of enteric origin in seawater, were initiated with typhoid bacillus (Izquierdo-Vicuña, 1981). By the end 1990, there were significative advances about *S. typhi* (back then *Bacillus typhi*) inability to survive for long time in coastal waters. Austin and Austin (1999), have demonstrated the presence of pathogenic enterobacteria in fish that lives in contaminated marine water by domestic wastewater, such as: *Escherichia coli, Enterobacter cloacae, Citrobacter* sp.*, S paratyphi A and B, S. enteritidis, S. amsterdam, S. give, P. vulgaris, P. rettgeri, Proteus mirabilis, Proteus morganii, Clostridium botulinum*, C group *Enterobacter*, *Streptococcus faecalis* and *Strptococcus faecium.* It has been proved the presence of *E. coli* and *S. aureus* and several species of *Salmonella* sp., in processed fish products (Rani *et al*. 2014).

The aim of this study was to a Rani *et al.* (2014). Ascertains if there is a significant difference in bacterial load of fish muscle of commercial interest, at the moment of their capture and after being processed for their sell.

Material and Methods

This study took place in eight different touristic and fishing zones of Mexican Caribbean: Isla Contoy, Isla Mujeres, Cancún, Puerto Morelos, Playa del Carmen, Isla Cozumel, Tulum and Ascension Bay, during July and August 2008. Twenty organisms from eleven different species that are considered commercially important were obtained from each zone, mentioned before: 10 recently captured fish, uneviscerated and without any consumption process; and 10 already processed for their commercialization in local markets and cooperatives. All the samples were refrigerated at 4ºC and transported in sterile conditions to laboratory for microbiological analysis.

In sterile field, the body surface of each fish was cleaned with soaked cotton with alcohol. Subsequently the dissection was made with a lateral cut, with sterile scalpel from operculum to caudal fin base. With abdominal area exposed, a sample of 10g of muscle was extracted and homogenized with 90 mL of sterile distilled water during three minutes, with a Virtix brand homogenizer. Dilutions from 10^{-1} to 10^{-7} were made (APHA, 1992), from each jar of dilution it was extracted, with an automatic pipette, 1000 µL and inoculated by duplicates in agar plates of specific environment of: Salmonella-Shigella (SS), eosin methylene blue (EMB): thiosulfate citrate bile salts (TCBS) and brain heart agar (BHA), and were incubated at 36ºC during 24 hrs, for count of colony forming units per milliliter (CFU mL^{-1}) in each dilution. From the jar with the original homogenized content, 1000 µL of sample was transferred to tubes with lactose and tetrathionate broth, to which 1000 µL of iodine were added, and also it was transferred to tubes with peptone water. The tubes were incubated at 36ºC during 24 hrs. From the bacterial growth that was obtained in Petri dishes, consecutive seeding in nutritive agar was made for the obtainment of pure colonies, which was confirmed by the homogenic growth in nutritive agar and by observation of colonies with a phase contrast microscope. Once confirmed the purity of the strains, Gram stain was made.

Colonies were identified according to Merck (1994) criteria and finally, in order to confirm the identification, all colonies were identified with the commercial kit API-20E (API, 1997). The whole procedure was carried out with collection strains: *Aeromonas hydrophila* (ATCC356), *Aeromonas caviae* (ATCC154), *Vibrio alginolyticus* (ATCC177) and *Vibrio parahaemolyticus* (ATCC178).

Results

The eleven fish species that were obtained, both at the moment of capture and in the different commercialization sites of the product were: *Lutjanus synagris* (pargo)*, Larimus argenteus* (boquinete), *Epinephelus morio* (mero), *Seriola dumerili* (coronado), *Haemulon plumieri* (chaecchi), *Scomberomorus maculatus* (sierra), *Calamos pennatula* (mojarra), *Caranx crysos* (cojinuda), *Mycteroperca bonaci* (abadejo)*, Mugil curema* (lisa), *Lutjanus campechanus* (huachinango) (Castro-Aguirre, 1976), obtained in the eighth different

sampling zones: Isla Contoy, Isla Mujeres, Cancún, Puerto Morelos, Playa del Carmen, Isla Cozumel, Tulum and Ascension Bay, where in each one it was obtained a different number of individuals : 50 *Lutjanus synagris* (pargo biajaiba) in seven of the zones; 30 *Epinephelus morio* (mero) in all zones; 19 *Larimus argenteus* (boquinete) in five zones; 24 *Seriola dumerili* (coronado) in three zones; 7 *Haemulon plumieri* (chaecchi) in three zones; 4 *Scomberomorus maculatus* (sierra) in one zone; and the species *Calamos pennatula* (mojarra) (2 organisms), *Caranx crysos* (cojinuda) (3 organisms), *Mycteroperca bonaci* (abadejo) (11 organisms)*, Mugil curema* (liseta) (2 organisms) and *Lutjanus campechanus* (huachinango) (one organism); each specie in one zone each, with a total of 152 analyzed organisms. The dominant fish species were: pargo, mero, boquinete and coronado; but the ones with higher consumption were: mero, pargo, huachinango and boquinete (Tab. 1).

Tab. 1: Fish collected of Mexican Caribbean

Locality	Specie	No. individuals	
		f	p
Puerto Morelos	*Lutjanus synagris*	6	3
	Haemulon plumieri	4	0
	Epinephelus morio	0	7
Playa del Carmen	*Larimus argenteus*	3	4
	comberorus maculatus	4	0
	Epinephelus morio	1	0
Cancún	*Lutjanus synagris*	0	4
	Mycteroperca bonaci	2	0
	Epinephelus morio	6	1
	Lutjanus synagris	2	8
Isla Cozume	*Larimus argenteus*	0	1
	Mycteroperca bonaci	9	0
	Seriola dumerili	0	10
	Epinephelus morio	1	0
Isla mujeres	*Seriola dumerili*	10	4
	Lutjanus synagris	0	3
	Epinephelus morio	0	3
Asencion Bay	*Haemulon plumieri*	0	2
	Epinephelus morio	3	0
	Lutjanus synagris	7	7
Tulum	*Larimus argenteus*	0	1
	Epinephelus morio	0	3
	Lutjanus campechanus	0	1
	Lutjanus synagris	1	5
Isla Contoy	*Larimus argenteus*	5	5
	Caranx crysos	0	3
	Lutjanus synagris	4	2
	Epinephelus morio	5	0
	Haemulon plumieri	1	0
	Calamos pennatula	0	2
	Mugil curema	0	2

- f: fresh fish; p: processed fish

In all the organisms of both types of samples that were obtained in the eight zones, it was registered a bacterial contamination. Zones where fish was registered with a higher bacterial load were: Cancun, Playa del Carmen and Ascension Bay. The number of identified bacterial species increased in five of the studied zones (62.5%), decreased in two zones (25%), and remained the same in one zone (12.5%), although the difference was not considered significant when applying a panel statistical analysis.

The diversity of isolated bacteria, increased in mojarras, cojinudas, chaecchis, boquinetes, lisetas, pargos, huachinango, abadejos and lisas, which represents 81.8% of the studied fish species, and remained the same in sierras and coronados, even though, $t_{obtained}$ (Tab. 2), indicates no significant difference in bacterial contamination levels between sampled organisms before and after the process of commercialization in the eight zones. In the eleven species of fish, there is not significant difference in bacterial load, regarding the number of different isolated species of fish before and after the process of commercialization (Tab. 2).

Tab. 2: Bacterial load difference of fish before and after being the capture.

Difference	$t_{obtained}$	$t_{calculated}$	Freedom degrees
Bacterial load of 11 sp. of fish in the 8 sampling zones	-44	2.3	7
Number of bacterial species in the 11 fish species	-3.6	3.1	10
Bacterial groups	0.63	2.8	18
CFU mL^{-1} quantified in S-S agar	2.8	3.1	10
CFU mL^{-1} quantified in EMB agar	2.56	3.1	10
CFU mL^{-1} quantified in TCBS agar	0.1	3.1	10
CFU mL^{-1} quantified in BHI agar	1.7	3.1	10

Quantitative analysis measured with colony forming units (UFC/mL^{-1}) from the bacterial load in different samples of fresh fish, registered quantities that depending in the isolation environment, it varied in the next way: S-S agar it was counted between a range of $2x10^2$ to $9x10^3$ CFU mL^{-1}; in EMB agar between 5 - $7x10^3$ CFU mL^{-1}; in TCBS between 0 - $8x10^{-1}$ CFU mL^{-1}; in BHI between $4x10^2$- $4x10^4$ CFU mL^{-1}; the bacterial load increased in processed fish samples, in the following amounts: in S-S between

$3x10^2$ and $7x10^4$ CFU mL^{-1}; in EMB between $2x10^2$ and 9 $x10^3$ CFU mL^{-1}; in TCBS between 0 and $2x10^2$ CFU mL^{-1}; and BHA between $4x10^4$ - $4x10^6$ CFU mL^{-1} (Tab. 3).

ab. 3: Quantitative analysis (CFU mL-1) of bacterial load of fresh fish and processed fish of Mexican Caribbean

Host	Culture media (Count ufc/mL^{-1})							
	S-S		EMB		TCBS		BHI	
	f	p	f	p	f	p	f	p
Calamos pennatula (mojarra)	$2×10^2$	$3×10^3$	$5×10^1$	$7×10^3$	$2×10^1$	$6×10^1$	$4×10^4$	$9×10^4$
Caranx crysos (cojinuda),	$2×10^3$	$4×10^4$	$4×10^2$	$7×10^3$	0	$1×10^1$	$4×10^5$	$4×10^6$
Epinephelus morio (mero)	$5×10^3$	$3×10^4$	$5×10^3$	$7×10^3$	$2×10^1$	$2×10^1$	$1×10^5$	$7×10^5$
Haemulon plumieri (chaecchi)	$1×10^3$	$7×10^4$	$3×10^2$	$7×10^3$	$5×10^1$	$2×10^2$	$6×10^5$	$4×10^5$
Larimus argenteus (boquinete	$3×10^3$	$3×10^4$	$5×10^2$	$9×10^3$	$3×10^1$	0	$3×10^5$	$4×10^5$
Lutjanus campechanus (liseta)	$8×10^2$	$1×10^4$	$4×10^2$	$7×10^3$	$8×10^1$	$2×10^2$	$7×10^5$	$4×10^6$
Lutjanus synagris (pargo)	$4×10^3$	$3×10^3$	$6×10^2$	$7×10^3$	$2×10^1$	$1×10^2$	$4×10^4$	$1×10^5$
Mycteroperca bonaci (abadejo)	$2×10^3$	$3×10^4$	$5×10^1$	$7×10^3$	0	4×10	$1×10^5$	$4×10^5$
Mugil curema (lisa)	$4×10^3$	$3×10^4$	$1×10^3$	$7×10^3$	$1×10^1$	$5×10^1$	$4×10^4$	$3×10^5$
Scomberomorus maculatus (sierra)	$9×10^3$	$1×10^4$	$8×10^2$	$7×10^3$	$4×10^1$	$2×10^2$	$8×10^3$	$4×10^5$
Seriola dumerili (coronado)	$9×10^3$	$3×10^4$	$7×10^3$	$9×10^4$	$3×10^1$	$2×10^2$	$1×10^5$	$4×10^5$

- S-S= Salmonella- Shigella, EMB= Eosin Methylene Blue, TCBS= Thiosulfate Citrate Bile Salts Sucrose, BHI= Brain Hearth Infusion
- f: fresh fish; p: processed fish

All of the identified bacterial flora from analyzed fish, regardless if it was fresh or processed, registered a presence of 19 different species of bacteria: 14 genus of: *C. freundii*, *C. amalonauticus* , *E. coli I*, *E. coli* , *E. coli III*, *Prot. miriabilis*, *Prot. rettgeri*, *Prot. vulgaris*, *Klebsiella flexneri*, , *S. typhi*, *S. thiphimurium*, *S. paratyphi*, *S. enteritidis*, *S. scochttmuelleri,* pertaining the Enterobacteriaceae family; on genus of *A. hydrophila*, from Aeromonadaceae family; three genus of Vibrio: *V. fluviales*, *V. cholera El Tor* and *V. parahaemolyticus*, from Vibrionaceae family; and finally, a specie from Pseudomonadaceae family , *Ps. fluorescens* (Tab. 4).Four species (21%) that were *A. hydrophila, E. coli III, Ps. fluorescens* and *V. fluviales*, registered presence in all the hosts (100%), and in both types of samples, while nine species (52.6%) increased their presence: *C. freundii, C. amalonauticus, Prt. miriabili, Prt. rettgeri, K. flexneri, S. tuhiphimurium, S. paratyphi, S. schottmuelleri*, *S. enteritidis* and *V. cholera El Tor;* seven bacterial species (36.8%) remained with the same number of hosts: *A. hydrophila, E. coli I* y *II, Prt. vulgaris, Ps. fluorescens, S. typhi* and *V. fluvialis;* only two species decreased: *V. cholerae EL Tor* and *V. parahaemolyticus.*

Discussion

Registered bacterial species in fresh and processed fish samples, warn high risk situation for human health due to ingest of fish, especially when it is consumed raw or with short baking time, and also because the presence of enterobacteria was predominant. All 19 identified species are considered as pathogenic for humans but also for aquatic organisms, like: *A. hydrophila* which has been experimentally proved its capacity as an opportunistic pathogen, rather cosmopolitan, that cause septicemia, and it has been associated with *A. salmonicida* as a causal agent of furonculose. In humans it is associated to gastroenteritis clinical conditions signs, caused by the ingestion of marine raw food; and also associated with *Citrobacter freundii,* that it is considered as a strict pathogen for aquatic species, mainly marine species (Roberts, 1999).

Salmonella medical importance is due to the high variety of diseases that they cause, that is going to depend on specie and serotype: *S. hirschfeldii* is a causal agent of bacteremia without intestinal

Tab. 4: Identified bacterial species in fresh and processed fish species at the coast of Yucatan.

Identified Pathogen	FISH																					
	A		B		C		D		E		F		G		H		I		J		K	
	f	p	f	p	f	p	f	p	f	p	f	p	f	p	f	p	f	p	f	p	f	p
Aeromnas hydrophila	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrobacter freundii	+	+	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-
Citrobacter amalonauticus	+	+	-	-	-	+	-	-	+	+	-	+	-	+	-	+	-	+	-	+	-	+
Escherichia coli I	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Escherichia coli II	+	+	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+
Escherichia coli III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Proteus miriabilis	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
Proteus rettgeri	+	+	-	+	-	+	-	-	+	+	-	-	-	+	-	+	-	+	-	-	+	+
Proteus vulgaris	-	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-
Klebsiella flexneri	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+
Pseudomonas fluorescens	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Salmonella typhi	+	+	-	-	+	-	+	+	-	-	-	-	+	-	+	-	-	-	+	-	+	-
Salmonella thiphimurium	-	+	+	+	-	-	+	+	+	+	-	-	+	+	-	-	-	+		-	+	+
Salmonella paratyphi	-	+	-	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	-
Salmonella enteritidis	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Salmonella scochttmuelleri	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
Vibrio fluvialis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vibrio cholerae El tor	+	-	-	-	+	+	-	-	+	-	+	+	-	-	+	+	-	-	+	-	-	-
Vibrio parahaemolyticus	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-	+	+	+	+

- A: *Calamos pennatula* (mojarra), B: C*aranx crysos* (cojinuda), C: *Epinephelus morio* (mero), D: *Haemulon plumieri* (chaecchi), E: *Larimus argenteus* (boquinete), F: *Lutjanus campechanus* (liseta), G: *Lutjanus synagris* (pargo), , H: *Mycteroperca bonaci* (abadejo), I: *Mugil curema* (lisa), J: *Scomberomorus maculatus* (sierra) K: *Seriola dumerili* (coronado)
- f: fresh fish; p: processed fish

participation; *S. schottmuellerie* cause paratyphoid fever; *S. paratyphi* A, also causes paratyphoid fever, characterized by gastroenteritis and bacteremia, this disease is normally transmitted by ingestion of contaminated food, in this case fish muscle; *S. typhi*, cause typhoid fever, characterized by being a generalized infection, transmitted by the ingestion of contaminated water and food (including fish and shellfish), and in general by healthy or sick carriers; *S. enteritidis* and its serotypes, which in general are etiological agents of salmonellosis. The common habitats of the previous species are feces of healthy or sick carriers of microorganism. Many investigations consider salmonellas as fecal contamination indicators in marine fish and shellfish, due to abundance with which they are presented and the importance of diseases they cause (Beach *et al.,* 2002; Zhao *et al.,* 2001).

The genus *Proteus,* was registered with three representatives in studied samples, *P. miriabilis, P. rettgeir,* and *P. vulgaris*, and was the second place in abundance between the enterobacteria. It constitutes an opportunistic group, because of their capacity to grow in "waves" in almost every environment, it is possible that it was found as a representative group. Also because of the advantages that their growth presents, sometimes it does not allow the growth of the desired culture. The three identified species, are considered as opportunistic pathogens in case of urinary, bacteremia and other pathological diseases. Some investigations involve *Proteus* genus as indicator of fecal contamination in marine fish, although their use as indicators is not very common (ILSI, 2011).

Members of *Citrobacter* were identified, represented by species *C. freundii* and *C. amalonaticus,* common inhabitants of water and food, and also of human intestine. This genus sporadically appears as opportunistic pathogens, it has been catalogued as "causal", when they have been identified in urinary tract and gastroenteritis diseases, which is why they are considered as opportunistic

intestinal pathogens, meaning they can be pathogens when the right conditions are present. It is catalogued as a low public health significance, because it is detected in lower numbers in feces, despite this, their major importance is in the capacity of multiply in contaminated water (Breeuwer *et al*., 2003)

Despite of being considered as the specie more associated to fecal in contaminated water environment, *Escherichia coli* presence in analyzed samples was very low. It is a normal member of native intestinal tract flora in humans and other animals, but it can be pathogenic, causing diseases like gastroenteritis. Identified species does not take part as normal flora of marine fish and their presence is basically due to marine water contamination with domestic wastewater and manipulation process of fish during capture, transport, preparation and elaboration for their consumption.

Bacteriological analysis showed that fish are already contaminated by pathogenic bacteria of human and animal origin from their natural environment, and that handling during the commercialization process does not significantly increase the contamination on them. It was observed that *S. hirschfeldi, S. schottmueleri, S.paratyphi A,* and *C. freundii*, presented a higher presence in processed fish samples, this behavior can be attributed to two important aspects: 1) The staff that handles the product since the capture until the sale can be healthy carriers of these pathogens, acting as vector and transmitter towards the products that are handled, increasing their number; and 2) bacteria contained in intestine, skin and gills of the fish, can increase when the fish dies (Olgunoğlu.2012)

Recently captured fish in the eight localities presented pathogenic bacteria contamination in a higher or lower grade. Localities with the highest contamination percentages (80-90%) were: Cancun, Playa del Carmen and Ascension Bay. Nevertheless, despite the high contamination levels, it is relative to take a free-living organism to decide whether a locality near other is contaminated or not, because the fish feeds and lives in the zone of its capture and surrounding waters, for this reason it is considered that identified bacterial contamination in samples of fresh fish is representative to the study zone and not of a locality in particular (Romero y Negrete, 2011).

Also in the market samples a presence of pathogenic bacteria was detected in all localities. The highest percentages (90-100%) were presented in Ascension Bay, Playa del Carmen and Tulum, these zones also presented high incidence in fresh fish samples, which shows that in these localities, fish in their natural environment and during their process for human consumption, have a higher pathogenic bacterial load than in surrounding localities.

Bacterial incidence was not selective in fish, because it was not presented in a higher or lower tendency in certain bacterial species, to contaminate just a certain species of fish. In the study zone there are important squama fisheries, which can bring serious public health issues, because fish catch in the zone represents an important source of food for regional consumption for locals and tourists and in a lower proportion at national and international level.

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