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Evaluation the Microbial Spoilage of Atlantic Salmon (*Salmo salar*) Fillets during the Packaging Processes and Its Control by Preservatives

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ABSTRACT

Seafood consumption is becoming increasingly popular due to the presence of high quality proteins, polyunsaturated fatty acids, and vitamins. Microbial fish spoilage is an area of global concern as it has been estimated that as much as 25% of all fish produced is lost post-harvest owing to microbial activity. This study examined the dynamics of microbial growth in fresh chilled Atlantic salmon (*Salmo salar*) packed in a modified atmosphere. Atlantic salmon were imported, handled, transported, and processed under optimal conditions. In the present work ten fillets (not smoked) and smoked fish samples were brought out from three different industrial steps in the Egyptian company for smoked salmon, El-Obour industrial area, Qalyubia Governorate, Egypt. The bacterial load (Total aerobic bacterial count) of these samples was determined as $4x10^4$ and $3x10^3$ cfu/gm for fillet and smoked samples respectively, this indicate that, all the investigated samples are in the acceptable range one million CFU for fillet and one hundred sousand CFU for smoked according to international standards ISO 4833/2003. Isolation of the contaminant microbiota results in obtaining of two different bacterial species *Bacillus cereus /thuringiensis* and *Citrobacter freundii* which identified by morphological and biochemical methods. To control the growth of these bacteria, various water plant extracts of different plant parts were assayed against these bacteria. Among the tested plant extracts, only extract of rosemary (*Rosmarinus officinalis*) showed strong antibacterial activity against *Citrobacter freundii*, FIM-SH and *Bacillus cereus /thuringiensis*, ZF with mean diameter of inhibition zones 16 and 29 mm respectively. These results demonstrate the potential for using plant extracts, especially rosemary extracts, as successful antibacterial agent. These extract could be used extensively as salmon additives in salmon products to reduce or eliminate salmon borne bacterial pathogens and to extend the shelf life of the packaged salmon samples.

Keywords: Seafood; Microbial Spoilage; Rosemary; Antimicrobial and Antioxidant

I. INTRODUCTION

Fish constitute the cheapest source of animal protein in Africa. It is one of the main food components of humans for many centuries and still constitutes an important part of the diet of many countries. The advantage of fish as a food resulted from its easy digestibility and high nutritional value (Clucas and Ward, 1996).

Fish consumption including fresh raw fish has increased due to the findings provided by nutrition and food science. Salmon (*Salmo salar*) is among the fish whose consumption as a fresh raw food has gradually increased, mainly presented as sushi and sashimi. As a result, hygienic-sanitary quality should be a matter of greater concern for this kind of food consumption as exposing consumers to different pathogenic microorganisms might lead to simple gastroenteritis and even death (Huss et al., 2000; Sato et al., 2005). Tuna and salmon are the most commonly consumed seafood and often consumed as raw, so it is significant to obtain the baseline information of bacterial community profile that could impact fish quality and public health about these two fish types (Nesheim and Yaktine, 2007).

The spoilage process in fish is well-documented and consists of autolytic degradation by fish enzymes and the production of unpleasant odors and flavors as a result of microbial action (Gram & Huss, 1996). Typically in the chilled seafood supply chain microbial mediated changes dominate the spoilage process (Emborg et al., 2002; Huss, 1995). The bacteria

responsible for spoilage in marine fish vary according to the harvest environment, the degree of cross contamination and the preservation methods applied post-harvest. The primary spoilage bacteria in aerobically packed fish are from the genera *Pseudomonas* and *Shewanella* while in modified atmospheres, *Photobacterium* as well as lactic acid bacteria (LAB) such as *Lactobacillus* and *Carnobacterium* are responsible for spoilage (Dalgaard et al., 1993; Emborg et al., 2002). Recent work also suggests that *Hafnia alvei* might also be a specific spoilage organism for modified atmosphere packed (MAP) Atlantic salmon (Macé et al., 2013).

Kvenberg (1991) and Rodeick (1991) classified the pathogenic bacteria associated with fish into the nonindigenous pathogenic bacteria and the indigenous pathogenic bacteria. The non-indigenous pathogens contaminate fish or fish's habitat in one way or the other and the pathogens include *Clostridium botulinum*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* species, *Shigalla* species and *Escherichia coli*, etc. The indigenous pathogenic bacteria are those naturally living in the fish's habitat. They are the *Vibrio* species and *Aeromonas* species etc. Several fish products are subjected to a mild heat treatment (equalling pasteurisation) and spore-forming bacteria (*Clostridium* or *Bacillus*) may grow in such products, particularly if unsalted (e.g. products cooked in vacuum pouches, i.e. sous vide products) (Ben-Embarek, 1994).

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertbutylhydroquinone (TBHQ) can control oxidation in foods, but the use of such compounds has been related to health risks (L¨oliger and Wille, 1993; Karpińska, 2014). Increasing consumer concern over the safety of synthetic preservatives and consumer preference for natural products have led to increased research on antioxidants derived from various natural sources such as cacao, rice, apple, red onion, oregano, licorice, rosemary, honey, and propolis (Geckil et al., 2005; Jiang et al., 2013).

Propolis is a natural product that is collected from certain plants by bees and contains various chemical compounds such as polyphenols (flavonoids, phenolic acids, and their esters), phenolic aldehydes, alcohols and ketones, quinones, steroids, and amino acids (Chaillou and Nazareno, 2009). The present study was carried out to evaluate the potential of the microbial spoilage in the Atlantic salmon (*Salmo salar*) fillets during the packaging steps in one of the Egyptian company for smoked salmon production and evaluating the using of natural plant extracts as antimicrobial agents and as applicable preservatives in the production processes.

II. METHODS AND MATERIALS

2.1 Study area and sampling

This study was carried out at the Egyptian Company for smoked salmon located in El-Obour industrial area, block 13018, plot 2, Qalyubia Governorate, Egypt. Ten frozen fish samples were bought from different production process as flow chart of process during the early morning hours of the day (between 8:30 and 10:00 am) when the fish was being brought out from cold room from different steps of work and different worker which were later transferred to the laboratory for biological assays.

2.2 Preparation of samples

Sample preparation was made using the method described by Obi and Krakowiaka (1983). About 10 g of the fish sample was cut from the head, middle and tail regions with a sterile knife. The cut samples were crushed into small pieces in a sterile mortar with about 10 ml sterile water. From the crushed sample, 1 ml aliquot volume was measured out and homogenized in a clean, dry sterile beaker containing 9 ml of distilled water giving a 1:10 dilution. This was done for the 10 fish samples

2.3 Enumeration of the microbial flora of the tested products

The bacterial load was estimated using the method described by Collins et al. (1989) and according to Egyptian standard 2760/PART1,2,3 for microbial count of frozen foods and involves the following:
Nine milliliters of sterile water was poured aseptically into five tubes each and 1 ml of the original crushed fish sample was added to the first test tube and mixed thoroughly. Another 1 ml was taken from the first tube and added to the second test tube and mixed very well. From the second test tube, another 1 ml was taken and introduced into the third test tube and mixed very well. This procedure continued until the fifth test tube. The crushed sample was therefore diluted from 10^{-1} to 10^{-5} for each fish sample.

Duplicate plates of nutrient agar were inoculated with 0.1 ml of the diluted solution (10^{-2} to 10^{-5}) using glass spreader technique. All plates were incubated at a temperature of 37°C for 24h before colony enumeration and isolation. The temperature was chosen to differentiate the mesophiles which constitute most important pathogenic bacteria (Baker and Silverton, 1985).

2.4 Isolation of microbiologic contaminants of the tested samples

For the purpose of isolation of growing microbial contaminants of the tested products each batch of the two products fillet (not smoked) and smoked fish were further inoculated by pour plate method into sterile petri dishes for each media (nutrient agar, MacConkey agar, tryptone soy agar and *Salmonella-Shigella* agar) prepared respectively, then incubated at appropriate incubation conditions

2.5 Characterization of the isolated microorganisms

2.5.1 Morphological charcateristics

The two isolates were subcultured and Gram staining was carried out. Identification of isolates was carried out based on the method described by Sakazaki and Shimad (1986), Collins et al. (1989) and Cheesbrough (2002).

2.5.2. Biochemical characteristics (BIOLOG ID System)

The technique a redox system BioLog is patented redox chemistry makes use of different carbon compounds including sugars, carboxylic acids, amino acids and peptides to provide an unparalleled wealth of discriminating biochemical characterizations. Biolog's powerful carbon source utilization technology accurately identifies pathogenic microorganisms by producing a characteristic pattern or "metabolic fingerprint" from discrete test reactions performed within a 96 well microplate. Culture suspensions are tested with a panel of pre-selected assays, then incubated, read and compared to extensive databases of environmental organisms, human pathogens, veterinary pathogens and plant pathogens.

Scope of the 96 assay reactions, coupled with sophisticated interpretation so ware, delivers a high level of accuracy that is comparable to molecular methods. one minute per sample set up is much simpler and faster than DNA sequencing and the automated pattern matching eliminates the need for training and expertise in gene sequence interpretation the method described by Biolog 2011. Illustration figure of Biolog is represented in figure (1).

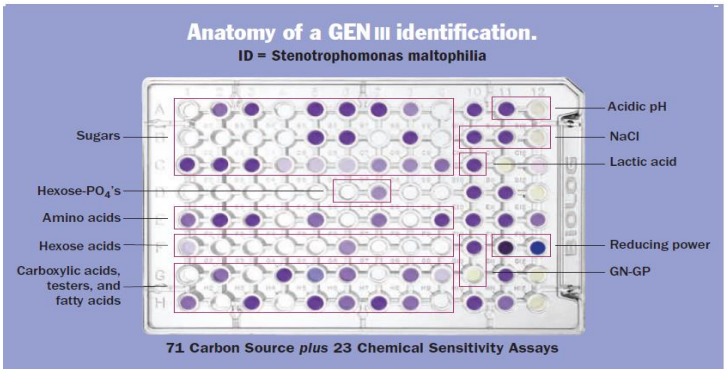


Figure (1): Anatomy of BIOLOG

2.6 Effect of different plant extracts on growth of the microbial contaminants

2.6.1 Disk diffusion method.

Antibacterial activities of two different plant extracts; rosemary (*Rosmarinus officinalis*) and propolis were assessed using the paper disk agar diffusion method according to Sacchetti et al. (2005) and Rasooli et al. (2006). Each tested microorganism was set up 16hrs before the assays to reach the log phase of growth (optic density at a wavelength of 600 nm= 0.4 to 0.5). Molten agar (5 ml, 40 to 45°C) containing 0.1 ml of microorganism suspension (10^7 cfu/ml) was spread over the surface of agar plates containing appropriate medium of each microorganism and left to solidify. Absorbent disks (Whatman disk No. 3 of 6 mm diameter) were placed in the inoculated Petri dishes than impregnated with 10 µl of different plant extracts. The blend of rosemary and propolis was used to study the effect of the one-half of the amount of each compound (v/v) from each essential oil on growth bacteria. Before incubation, all the Petri dishes were kept in a refrigerator (4°C) for 2 hrs to stop the bacteria from multiplication. Then they were incubated at 37°C for 24 hrs and the diameters of the inhibition zones, including the 6 mm disk, were measured (mm) respectively, to determine the sensitivity of each bacterial species tested. All the tests were performed in triplicate.

III. RESULT AND DISCUSSION

3.1 Enumeration of microbial flora of the tested products

The salmon products under study were tested for their microbial numbers and types by the methods described previously in the part of material and methods through the following:

3.2 Total aerobic viable count

The results of the total microbial count of the tested products against bacteria, yeasts and molds were recorded in table (1) as the health ministry analysis. Data recorded in the above table showed that, the total aerobic bacterial count limits of the customer processed samples was $4x10^4$ and $3x10^3$ cfu/gm for fillet and smoked respectively. This result indicate that the investigated food samples according to international standards ISO 4833/2003 as the standard of aerobic plate count for RTE foods $\leq 10^5$ cfu/g, so all samples are accepted in which they are not exceed the standard of aerobic plate count limit. Aquino et al. (1996) found higher values pointing to a variation from $3.0x10^3$ to $2.5x10^7$ cfu/g. In comparison to the results obtained in the present study, such variations indicate a reasonable condition presented by frozen salmon as well as the importance of such conservation method in order to maintain the initial microbiological features of the product so as to avoid deterioration.

Table (1): The range of total microbial counts of the analyzed salmon samples

Sample code	Sample description	Bacterial count (cfu/gm)	Yeasts and molds count (cfu/gm)
TCF	Fillet whole side	$4x10^4$	0
FD	Dobree smoked no sliced sample	$30x10^3$	0
FS	Smoked sliced sample	$1x10^4$	0
ZF	Fillet from orsal side as loin	$4x10^4$	0
FIM-SH	Worker slice fillet	$5x10^3$	0

3.3 Isolation of microbiologic contaminants of the tested samples

The result of isolation of microbial contaminants of the tested products revealed that; growing of 10 bacterial isolates, while there is no yeast or molds contamination was obtained.

3.4 Characterization of the obtained bacterial isolates

The obtained bacterial isolates were identified to genus level. Based on preliminary tests, appropriate rapid identification systems and other biochemical analyses using Biolog were chosen. The result of identification of the obtained bacterial isolates revealed that a total of 2 biochemically distinct isolates were obtained from the tested products named as FIM-SH and ZF, Data of characterization of these isolates are in the following:

3.4.1. Morphological characteristics

Culture characteristics on nutrient agar (figure 2) showed smooth colonies, generally small 2-4 mm, moist and gray with shiny surface and entire edge for the isolate FIM-SH, on the other hand colonies of the isolate ZF were large, irregular, opaque, with a waxy aspect. Light microscopy investigations (figure 3) of Gram-stain showed Gram-negative straight rods, found singly and in pairs for the isolate FIM-SH while the isolate ZF was seen as Gram-positive bacilli found in diploid and in chains.

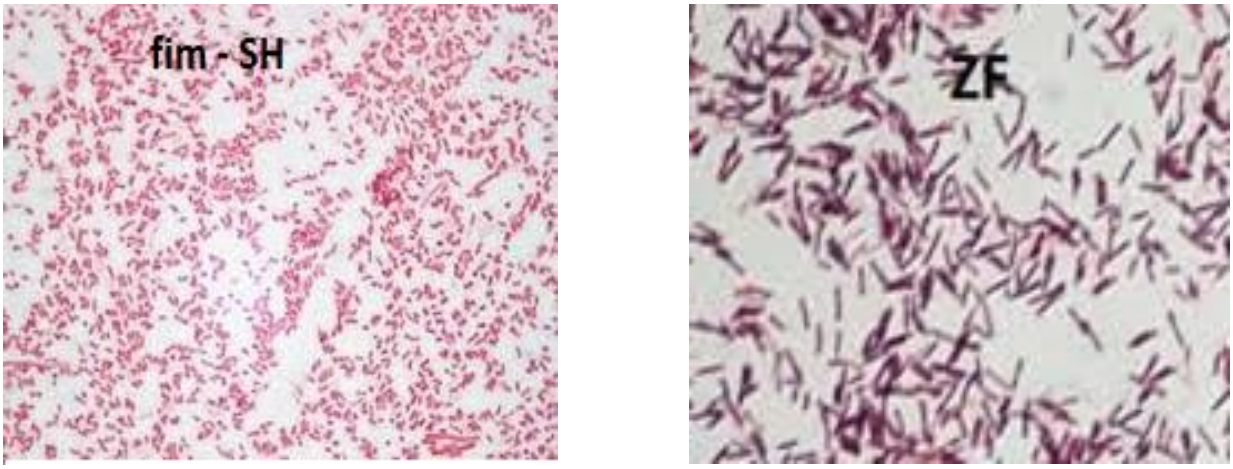


Figure (2): Light microscope photograph showing Gram stain reaction, cell shape and arrangement of FIM-SH and ZF bacterial isolates.

3.4.2. Biochemical characteristics

According to the results obtained from morphological and biochemical characteristics, the isolate (FIM-SH) was assigned to *Citrobacter freundii* and designated as *Citrobacter freundii*, FIM-SH, while the isolate (ZF) was assigned to *Bacillus cereus* /*thuringiensis* and designated as *Bacillus cereus* /*thuringiensis,* ZF. This finding is in agreement with the results of Moshood et al. (2012) that result the *Bacillus cereus* was found to have the highest frequency of occurrence in iced smoked fish samples, with 57% occurrence, when compared with the other isolates. *Staphylococcus aureus* had the highest frequency of occurrence in the dried smoked fish samples (DSF), with the value of 44%. In the dried smoked fish samples, *Bacillus cereus* had 31% occurrence, *Salmonella typhi* had 13%, *Klebsiella* spp and *Proteus mirabilis* both had 6% occurrence each. In the iced smoked fish samples, *Staphylococcus aureus* had 29% occurrence, *Klebsiella* spp and *Streptococcus* spp both had 7% occurrence each. These counts can generally be regarded as acceptable limits. However, they are considered as potentially hazardous as food with these levels (10^4 cfu/g) of contamination may result in food-borne illness if consumed.

Table (2): Biochemical tests based on BIOLOG technique for FIM-SH

No.	Test	Result	No.	Test	Result	No.	Test	Result
A1	Negative control	-	C9	Inoline	+	F5	D-Glucuromic acid	+
A2	Dextrin	±	C10	1%Sodium Lactate	+	F6	Glucuranamide	+
A3	D-Maltose	+	C11	Fusidic acid	+	F7	Mucic acid	+
A4	D-Trehalose	+	C12	D-Serine	+	F8	Quinic acid	-
A5	D-Cellobiose	+	D1	D-Sorbitol	+	F9	D-Saccharic acid	+
A6	Gentiobiose	+	D2	D-Mannitol	±	F10	Vancomycin	+
A7	Sucrose	-	D3	D-Arabitol	-	F11	Tetraxolium violet	+
A8	D-Turanose	-	D4	Myo-Inositol	-	F12	Tetraxolium blue	+
A9	stachyose	-	D5	Glycerol	+	G1	P-hydroxy-Phenyl	-
A10	Positive control	+	D6	D-Glucose-5-PO4	+	G2	Methyl Pyruvate	+
A11	Ph 6	+	D7	D-Fructose-6-PO4	+	G3	D-Lactic Acid Methyl	-
A12	Ph 5	+	D8	D-Aspatic Acid	±	G4	L-lactic Acid	+
B1	D-Raffinose	+	D9	D-Serine	+	G5	Citric Acid	+
B2	D-Lactose	+	D10	rondomycin	+	G6	A-Keto-Glutaric Acid	
B3	D-Maltose	+	D11	Rifamycin 8V	+	G7	D-malic Acid	+
B4	B-Methyl D-Glucosid	+	D12	minocycline	-	G8	L- malic Acid	+
B5	D-SAILICIN	-	E1	Gelatin	-	G9	Bromo – Succinic Acid	+

B6	N-Acetyl-D-	±	E2	Glycyl-L-prolline	+	G10	Nailc Lactic Acid	-
B7	N-Acetyl-B-D-	±	E3	L-Alanine	+	G11	Lithium Chloride	+
B8	N-Acetyl-D-	+	E4	L-Arginine	-	G12	Potassium Tallurite	-
B9	N-Acetyl-D-	+	E5	L-Aspartic acid	+	H1	Tween 40	-
B10	1% NaCl	+	E6	L-Glutamic acid	±	H2	ᵛ-Amino-Butyric Acid	-
B11	4% NaCl	+	E7	L-Histadine	-	H3	α-Hydroxy-Butyric	+
B12	6% NaCl	+	E8	L-Pyroglutamic	-	H4	β-hydroxy D,L-Butyric	+
C1	D-Glucose	+	E9	L-Serine	+	H5	α keto Butyric Acid	+
C2	D-Mannose	+	E10	Lincomycine	+	H6	Acetoacetic Acid	+
C3	D-Fructose	+	E11	Guanidine HCl	+	H7	Propionc acid	+
C4	D-Galactose	+	E12	Niaproof 4	+	H8	Acetic Acid	+
C5	3-Metyl Glucose	+	F1	Pectin	×	H9	Formic Acid	+
C6	D-Fucose		F2	D-Galacturonic	+	H10	axtreonam	+
C7	L-Fucose	+	F3	L-Galactonic acid-lactone	+	H11	Sodium Butyrate	+
C8	L-Rhaminose	+	F4	D-Gluconic acid	+	H12	Sodium Bromate	±

Table (3): Biochemical tests based on BIOLOG technique for ZF isolate.

No.	Test	Result	No.	Test	Result	No.	Test	Result
A1	Negative control	-	C9	Inoline	+	F5	D-Glucuromic acid	±
A2	Dextrin	+	C10	1%Sodium Lactate	+	F6	Glucuranamide	±
A3	D-Maltose	+	C11	Fusidic acid	-	F7	Mucic acid	-
A4	D-Trehalose	+	C12	D-Serine	+	F8	Quinic acid	-
A5	D-Cellobiose	±	D1	D-Sorbitol	-	F9	D-Saccharic acid	-
A6	Gentiobiose	-	D2	D-Mannitol	-	F10	Vancomycin	-
A7	Sucrose	+	D3	D-Arabitol	-	F11	Tetraxolium violet	-
A8	D-Turanose	-	D4	Myo-Inositol	-	F12	Tetraxolium blue	-
A9	Stachyose	-	D5	Glycerol	+	G1	P-hydroxy-Phenyl Acetic Acid	-
A10	Positive control	+	D6	D-Glucose-5-PO4	+	G2	Methyl Pyruvate	+
A11	Ph 6	+	D7	D-Fructose-6-PO4	+	G3	D-Lactic Acid Methyl Ester	±
A12	Ph 5	-	D8	D-Aspatic Acid	-	G4	L-lactic Acid	+
B1	D-Raffinose	-	D9	D-Serine	±	G5	Citric Acid	±
B2	D-Lactose	-	D10	Rondomycin	-	G6	A-Keto-Glutaric Acid	±
B3	D-Maltose	-	D11	Rifamycin 8V	-	G7	D-malic Acid	-
B4	B-Methyl D-Glucosid	-	D12	Minocycline	-	G8	L- malic Acid	+
B5	D-SAILICIN	-	E1	Gelatin	+	G9	Bromo – Succinic Acid	±
B6	N-Acetyl-D-Glucosamine	+	E2	Glycyl-L-prolline	±	G10	Nailc Lactic Acid	-
B7	N-Acetyl-B-D-Mannosamine	-	E3	L-Alanine	±	G11	Lithium Chloride	+
B8	N-Acetyl-D-Galactosamine	-	E4	L-Arginine	±	G12	Potassium Tallurite	+
B9	N-Acetyl-D-Muraminic Acid	-	E5	L-Aspartic acid	±	H1	Tween 40	±
B10	1% NaCl	-	E6	L-Glutamic acid	+	H2	ᵛ-Amino-Butyric Acid	-
B11	4% NaCl	+	E7	L-Histadine	+	H3	α-Hydroxy-Butyric Acid	-
B12	6% NaCl	+	E8	L-Pyroglutamic acid		H4	β-hydroxy D,L-Butyric Acid	±

C1	D-Glucose	+	E9	L-Serine	+	H5	α keto Butyric Acid	-
C2	D-Mannose	-	E10	Lincomycine		H6	Acetoacetic Acid	+
C3	D-Fructose	+	E11	Guanidine HCl	+	H7	Propionc acid	±
C4	D-Galactose	-	E12	Niaproof 4		H8	Acetic Acid	±
C5	3-Metyl Glucose	-	F1	Pectin	+	H9	Formic Acid	+
C6	D-Fucose	-	F2	D-Galacturonic acid	±	H10	axtreonam	+
C7	L-Fucose	-	F3	L-Galactonic acid lactone	-	H11	Sodium Butyrate	+
C8	L-Rhaminose	-	F4	D-Gluconic acid	+	H12	Sodium Bromate	+

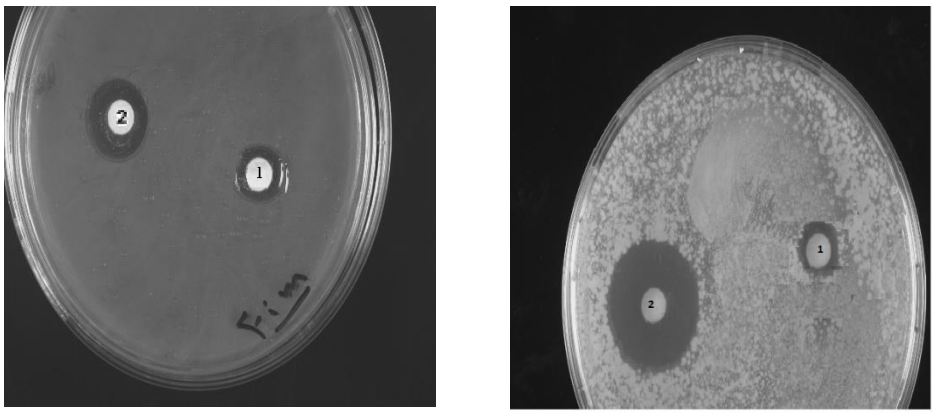
3.5 Effect of different plant extracts on growth of the microbial contaminants

Plant products, particularly spices and extracts of various plant parts have been used extensively as natural antibacterials and antioxidants. In the commercial preservation of fish and fish products, natural antioxidants from plant sources have been found to extend shelf life and prevent fishy taste and flavor (Pazos et al., 2008; Luther et al. 2007, Martos et al. 2007). In the present study, rosemary extract was found to be the most active extract with antibacterial activities on two bacterial species associated with salmon salar spoilage followed by propolise extract was found to low antibacterial activities on two bacterial species associated. Data of antibacterial activity are recorded in table (4) and illustrated in figure (4).

Table (4): Antibacterial activity of rosemary (*Rosmarinus officinalis*) leaves extract and propolis extract

No.	Bacterial organism	Antibacterial activity with mean diameter of inhibition zone (mm) of the most active plant extracts	
		Propolis	Rosemary
1	*Citrobacter freundii*, FIM-SH	13.0	16.0
2	*Bacillus cereus /thuringiensis*, ZF	11.0	29.0

In general, all the microorganisms associated with salmon salar spoilage were inhibited by rosemary extract used in this study. But Agatemor (2009) who found that hot water extracts of all plants tested inhibited all microorganisms, thus the efficacy of plant extracts evaluated as antibacterial agents was dependent on the solvent of extraction. The extracts of some Nigerian spices were more potent against common food borne microorganisms including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Streptococcus faecalis.*



(A) **(B)**

Figure (3): Antibacterial activity for extracts of propolis (disc no. 1) and rosemary (disc no. 2) against the fish contaminant bacteria; (A): *Citrobacter freundii*, FIM-SH and (B): *Bacillus cereus* / *thuringiensis*, ZF.

IV. CONCLUSION

The studied plant could provide some activity against the fish spoilage bacteria; however, it is not known that which component of the extract is responsible for this effect. Further studies using isolated constituents instead of the whole extract should be carried out. This study confirms the efficacy of water extracts of rosemary and their potential as organic preservatives in fisheries and aquaculture. Finally this research is the first for using rosemary extract as a crud on smoked salmon that have preservatives and flavor properties.

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