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Evaluation of Inhibitory Activity of some Natural Materials on Biofilms Formed on Fresh Fish Cutting Boards

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ABSTRACT

This research tried to evaluate the inhibitory activity of some natural, nontoxic, and inexpensive materials such as Iodized Salt (IS), Concentrated Natural Lemon Juice (CNLJ) and combination of both on one month old biofilm formed on $2cm^2$ of cutting board. The treatment period ranged from 24 to 72 hours and the results shows that the combination of (IS+CNLJ) for 24 hours have a cidal effect on all microorganisms that formed biofilm on cutting board. *Streptococcus pneumonae, E.coli, Klebsiella spp., Proteus vulgaris, Salmonella spp., Staphylococcus spp., Pseudmonas spp., Listeria spp. and Candida spp.* were isolated from cutting board, Knives, hands and inner surface of plastic container with different percentage ranging from 1.4%to 15.4%.

Keywords: Fresh fish biofilms, Fish cutting board, Lemon juice, Iodized salt.

تقييم الفعالية التثبيطية لبعض المواد الطبيعية على الأغشية الحيوية المتكونة على ألواح تقطيع الأسماك الطازجة

الملخص

شمل البحث محاولة تقييم الفعالية التثبيطية لبعض المواد الطبيعية غير السامة والرخيصة الثمن والتي تضمنت ملح الطعام باليود وعصير الليمون المركز ضد الأغشية الحيوية بعمر شهر واحد المتكونة على مساحة ($2سم^2$) من لوح تقطيع الأسماك الطازجة. تراوحت فترة المعالجة بين (24– 72) ساعة وأظهرت النتائج بأنّ مزيج ملح اليود مع عصير الليمون لمدة 24 ساعة كان له تأثيرٌ قاتلٌ لكل الأحياء المجهرية التي كوّنت الأغشية الحيوية.

تمّ في هذا البحث أيضاً عزل كل من *Streptococcus pneumoniae*، *E. coli*، *Klebsiella* spp.، *Proteus vulgaris*، *Salmonella* spp.، *Staphylococcus* spp.، *Pseudomonas* spp.، *Listeria* spp.، *Candida* spp. من ألواح تقطيع الأسماك والسكاكين وأيدي العاملين والأسطح الداخلية لحاويات الأسماك بنسب مختلفة تراوحت بين (1.4%) إلى (15.4%).

الكلمات الدالة: الاغشية الحيوية للأسماك الطازجة، ألواح تقطيع الأسماك، عصير الليمون، ملح الطعام مدعم اليود.

INTRODUCTION

Biofilms are collective of one or more types of microorganisms that can grow on many different surfaces. Microorganisms that form biofilms include bacteria, fungi and protists. (Vidyasagar, 2016).

In their natural environments, fish are exposed to myriad of microorganisms some of which compromise the shelf life of the product and/or safety in human (Martha *et al*., 2012). The contamination occurs naturally from the environment where fish are harvested, during harvesting, processing or during food preparation, while cross contamination occur during food processing and

preparation where bacteria are transferred from raw fish and/ or contaminated safe food, also contaminated water may introduce pathogen into foods (Wekell *et al.,* 1994).

The formation of a microbial biofilm on the surface of fish processing equipment increases the threat of a cross over contamination of the product (Kusumaningrum *et al*., 2003). Different fish processing establishment have different ways of cleaning and washing their equipment, water temperature, water hardness, acidity, surface material of equipment, type of detergent or disinfectant and their concentration are examples of variable that are likely different in each plant and between countries (Guobjornsdottir *et al.*, 2005).

In Iraq, there is no fish processing factories or plants, but there are many small shops with very simple equipments like small cutting boards (made of rubber), stainless steel knifes, the sellers store new harvested fish in a plastic containers and they use their necked hands without cloves, they did not use any detergent to clean these equipment just washing them with water, therefore our study aimed to screen about biofilm forming microorganisms and trying to treat formed biofilms on the cutting board, by using natural material (Lemon juice and Iodize salt) for the first time locally.

MATERIALS AND METHODS

Materials

Media: many selective and differential media were used in this study to isolate and identify the biofilm forming microorganisms, these media were: Blood agar (BA). Cetramide Agar, MacConkey Agar (MA), Sabroid Dextrose Agar (SDA), Manitol Salt Agar (MSA), Triple Sugar Iron Agar (TSIA), Salmonella-Shigella Agar (SSA), Peptone water, Urea Agar Base. Simon Citrate Agar, Carry Blair transport media (Commercial).

All these media were prepared as mentioned in (Cruikshank *et al*., 1975).

Gram's stain: prepared as described by Prescott *et al*., (1996)

Treatment materials

-Concentrated Natural lemon Juice (CNLJ) locally prepared.
-Iodized salt (Turkey) its contents:
1-Refined salt (Sodium Chloride)
2-Grounding preventer material (potassium ferrosiyanid e53).
3-Potassium iodide 25-40 mg/kg.
- Combination between (CNLJ and IS).

Methods

Samples collection

Forty (40) swabs were taken from fish contact surfaces including:
Cutting boards (made of rubber), Stainless steel knives, Sellers hands, Inner surfaces of plastic containers that used for new harvested fish preserving.

These samples were taken from ten fish sailing shops selected for this study.

Microbiological study

The samples were transported to the laboratory during one hour. All swabs were cultured on (BA), (MA), (MSA), (S.S.A.), cetramide agar and (SDA). The incubated aerobically at 37ᵃ for 24, and 48 hours.

Smears from colonies on each media were done and stained with gram' s stain and examined by light microscope with oil immersion lens.

Identification of isolates were done depending on biochemical test (TSI, IMVIC, urea production and oxidase test) as described by Steve and Dennis (2001).

Screening about biofilm forming microorganisms on cutting board and treatment method:

A. Biofilm forming microorganisms:

One cm^2 of cutting board was cut from one month used cutting board for screening about biofilm forming microorganisms. Samples were taken by using sterile cotton swab and cultured on

(BA), (MA), (MSA), (S.S.A), (SDA) and cetramide agar then incubated at 37°C for 24 and 48 hours and the results were recorded.

B. **Biofilm treatment:**

Three samples of $2cm^2$ from the same cutting board were taken and each one was immersed separately for 24, 48 and 72 hours as a period of treatment in:

1. 20 gram of iodized salt(IS)
2. 20 ml of concentrated natural lemon juice (CNLJ)
3. 20 gram of (IS)+20 ml of (CNLJ).

After every period of treatment the samples were washed thoroughly with sterile normal saline and screening test about survival microorganisms were done by using sterile cotton swabs and cultured on (BA), (MA), (MSA), (S.S.A), (SDA) and cetramide agar, incubated them at 37°C for 24 and 48 hours and the results were recorded.

RESULTS AND DISCUSSION

The results in Fig. (1 A, B, C, D) showed that the higher percentage of bacterial existence belong to *Streptococcus pneumoniae, E.coli, Klebsiella spp*. and *Proteus vulgaris* with 14.0% on cutting board, 14.4% on knives, 15.4 % hands and 14.0 % on The inner surface of plastic containers, followed by *Samonella spp*. 14.0%, 13.0 %, 15.4% and 14.0 on the same surface respectively while *Staphylococcus spp.* percent were 12.6%, 11.5%, 12.3% and 12.6% then *Pseudomonas spp*. 11.2%, 8.7%, 3.0% and 5.6%, followed by *Listeria spp*. with 1.4%, 2.8%, 1.5% 8.4% respectively.

Candida spp. showed a higher percent on hands 6.1%, then 5.8% on knives, while on cutting board it represent 4.2% and 2.8% on the inner surface of a plastic containers.

In fish processing plants, microorganisms could attach themselves on surfaces and form biofilms, in the presence of required nutrients, minerals and organic matter (Kariyawasam and Jayasooriya, 2006).

The results of multi species and genuses in this study are agreed with Goller and Romeo, (2008) when they referred that the biofilms in nature are generally multispecies, spatial and metabolic interaction between species contribute to the organization of multispecies biofilm and the production of dynamic local environment.

Bagge *et al.*, (2001) found that the materials of the surfaces were, Teflon , PVC, and PVDE (kynar) and anywhere that had continuous contact with products is possible especially where surfaces irregularities occur.

Cross contamination occurs when cells detached from biofilm structure once food passes over contaminated surfaces or through aerosols originated from contaminated equipment (Rodrigues *et al*. 2011), therefore the same microorganisms were isolated from cutting board, knives, hands , and plastic containers in this study.

Although the earliest reports on pneumococcal biofilms go back 10 years or more. The last 5 years have seen an increase in the number of studies examining pneumococcal biofilms at the structural and genetic level (Shikongon *et al.*, 2010). Campylobacter and Salmonella are leading causes illnesses worldwide, vastly harbored by raw meat as their common food reservoir. Both microbes are prevalent in meat processing environment in the form of biofilms that contribute to cross-contamination and food borne infection (Jiaqi *et al*., 2017).

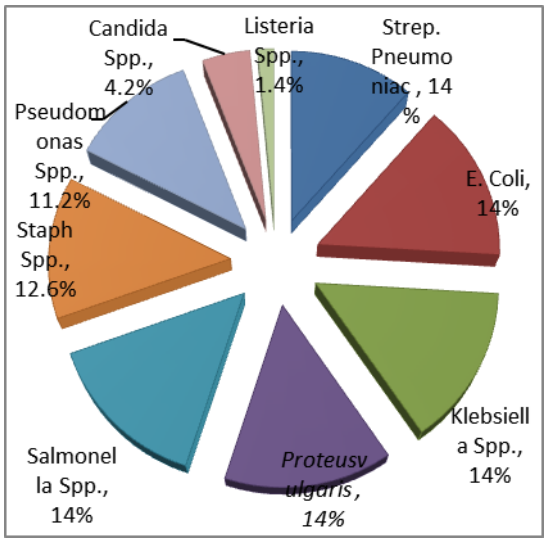
In biofilms bacterial pathogens can form part of biofilms and pose a challenge to public health and food shelf life as well as safety like *E.coli*, *Pseudomonas aeruginosa, S.almonella* spp. and *Listeria monocytogenes* (Reisner *et al*., 2006).

Brook and Flint (2008), Referred in their study that the poor sanitation of food contact surface is believed to be an essential contributing factor in food borne disease outbreaks, especially those involving *Listeria monocytogenes* and Salmonella spp.

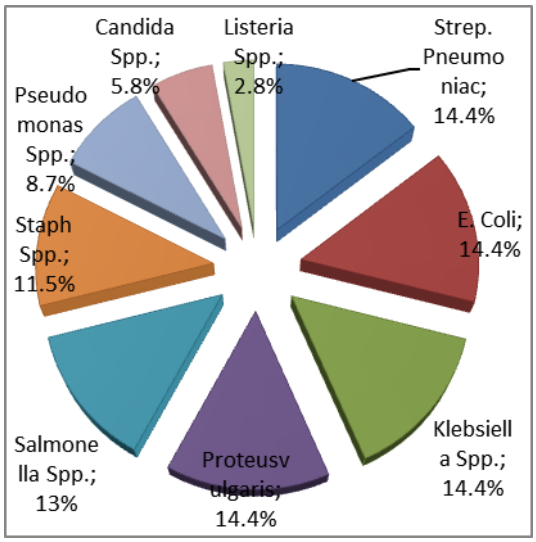
Reynisson *et al*., (2009) found that *Klebsiella spp.* Composed of 3% of species composition of cultured isolates from bacterial biofilms from fish processing surfaces while Moretro *et al*., (2003) found that Staphylococcus spp. are able to form biofilm on food and food processing environment.

About the existence of Candida spp. in the biofilm, Baillie and Douglas (1999) illustrated that the biofilm of *Candida albicans* usually consist of a mixture of yeast, hyphae, and pseudohyphae and may have a basal yeast layer that anchor the biofilm to the surface.

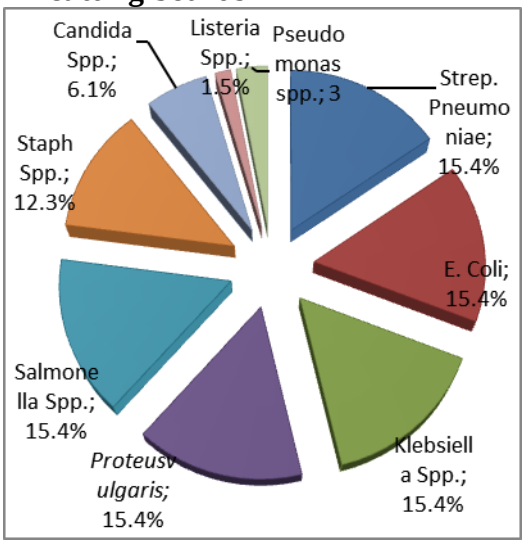
Some bacteria are able to form biofilm in isolation, while others establish synergistic association termed co-aggregates, in which one organism acts as a primary attachment candidate and exopolysaccharide producers providing a favorable environment and protection for the other species (Palmer *et al*., 2007).



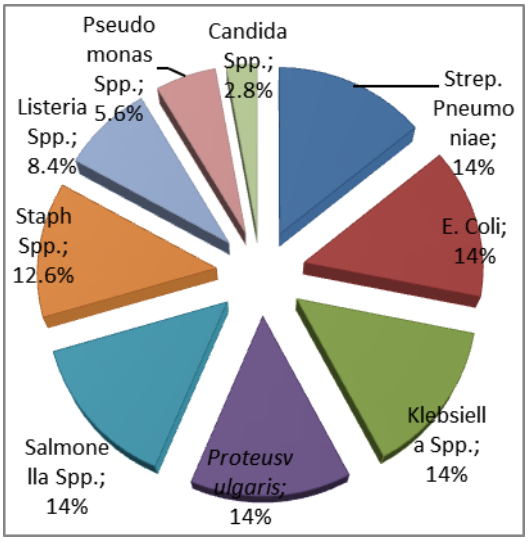
A: Percentage of bacterial existence on cutting boards



B: Percentage of bacterial existence on Knives



C: Percentage of bacterial existence on hands



D: Percentage of bacterial existence on the inner surface of a plastic containers

Fig. 1: Percentages of bacterial existence on different surfaces

The results in (Table1) showed treatment materials which used to treat a biofilm on cutting board with (IS), (CNLJ) and (IS+CNLJ) for three periods 24.48, and 72 hours.

As shown in Table (1) Listeria spp., *E.coli,* and Klebsiella spp. were grow after 24 hours of immersing the cutting board in (IS) and (CNLJ) separately, but, there was no growth after treatment with the combination of (IS+CNLJ) for the same period, also there was no growth for these bacteria after 48 and 72 hours of immersing in (IS) and (CNLJ) separately.

Proteus vulgaris and *Salmonella* spp. shows no growth after treatment for the three periods of immersing except the treatment (CNLJ) after 24 hours as it illustrated in (Table 1), this result may be also due to slow penetration of (CNLJ) to the bifilm layer and the high effect of (IS) through 24 hours on the cells of these microorganisms.

All microorganisms in this study could not grow (survive) after treatment with the combination of (IS) and (CNLJ) after 24 hours of immersing as it illustrated in table 1 and this result as it noted in Fig.(2) represented by 0% of microorganisms survival percent, this typical result may due to the mechanisms of antibiofilm treatment materials, starting with dehydration of the moisture nature of biofilm, osmotic effect of (IS), and the effect of the low pH of (CNLJ) on lipopolysaccahride and lipoprotein of microorganism.

In this study there was an attempt to remove a mature biofilm (one month old) from cutting board by using a natural, inexpensive, and available materials as a disinfectant.

It is necessary to clean and sanitize the equipment properly especially those that in contact with the product for a long period of time after each processing cycle to minimize the biofilm formation, pH of the acid solutions must be monitored below 2 to hydrolyze the polysaccharide coating that protect the biofilm (Edstrom, 2003).

Nack *et al*., (2009) found that weak organic acid like lactic acid and combinations are effective bacterial agents against planktonic gram negative and gram positive multiresistance bacteria, enhanced efficacy with gram negative bacteria is probably due to their lipopolysaccharide layer, which is more permeable to acids due to the acid soluble lipids components, and these weak organic acid alone or in combination are also capable to penetrate into biofilms, increasing their potential to be used as sanitizer.

All microbial biofilm consist of either a single layer of attached cells or may be defined three dimentional structure with species specific architectural organization that may or may not consist of microcolonies interspersed by water channels, the architectural of a mature biofilm depends on the hydrodynamics of the surrounding fluid (Chemielewski and Frank, 2003), therefore (IS) can control on this hydrodynamics by drying these water channels and this mechanism well be destroy the architecture of mature biofilm as well as osmotic effect of (IS) on microorganisms cells.

These results may be due to the old age of biofilm (one month old) which protect biofilm by slowing the penetration of treatment materials and when treatment periods were extended to 48 and 72 hours, these materials were penetrated the layer of biofilm and inhibit the growth of these microorganisms, and this is the advantage of using a natural components as antibiofilm material because there is no side effect on the products or human unlike the treatment with chemical or physical materials.

It is important to use lower concentration of chemical and physical disinfectant and short contact time in food, food plants, and water sanitation because of their side effect like leaving potential carcinogens in environment when chlorine, H_2O_2 and ozone used (Chawla, 2006).

Table 1: Treatment materials which used to treat a biofilm Forming M.O. on cutting board for three periods 24.48, and 72 hours

	Duration of treatment / hours						
	24			48		72	
Treatment / microorganisms	IS	CNLJ	IS+ CNLJ	IS	CNLJ	IS	CNLJ
Listeria Spp.	+	+	-	-	-	-	-
Staphylococcus Spp.	+	+	-	+	+	+	+
Streptococcus pneumonae	+	+	-	+	+	+	+
E. Coli	+	+	-	-	-	-	-
Klebsiella Spp.	+	+	-	-	-	-	-
Proteus vulgaris	-	+	-	-	-	-	-
Pseudomonas Spp.	-	+	-	-	-	-	-
Salmonella Spp.	-	-	-	-	-	-	-
Candida Spp.	+	+	-	+	+	+	+

IS: Iodized Salt, CNLJ: Concentrated Natural Lemon Juice
+: Growth, -: No growth.

In Fig. (2) the results of survival percentage showed high value 88.8% when (CNLJ) was used for 24 hours then 66.6% when (IS) was used for the same period.

These results were decreased to 33.3% when (IS) and (CNLJ) used separately for 48 and 72 hours, these results may be due to long contact periods between treatment material and biofilm matrix or due to some survival strategies that used from microorganisms to persist against treatment materials.

It has been suggested that such persistence is likely due to physical adaptation of cells in biofilms, particularly resistance to cleaning and sanitizing regimes, since it is generally accepted and well documented that cells within a biofilm are more resistance to biocides than their planktonic counter parts (Carpenter and Cerf, 1993).

Staphylococcus spp. was one of the surviving microorganisms (*Candida* spp. and *streptococcus pneumoniae*) and according to Moretro *et al.*, (2003), when they examined the ability of staphylococcus isolates from food and food processing environments to form biofilms, they found that these strains formed thicker layers of biofilm when sodium chloride or glucose was added to the medium. The other reason may be due to the type of extra polysaccharide (EPS) that supposed to be the main cement for cells in biofilm, the types of EPS vary from organism to other (Sutherland, 2001). It is strongly believed that the ability of *salmonella* spp. to form biofilms on inanimate surfaces contribute to its survival and persistence in non-host environments and its transmission to new hosts, to this direction (Vestby *et al.*, 2009) found a correlation between the biofilm formation capacity of III salmonella strains isolated from feed and fish meat factories and their persistence in the factory environment.

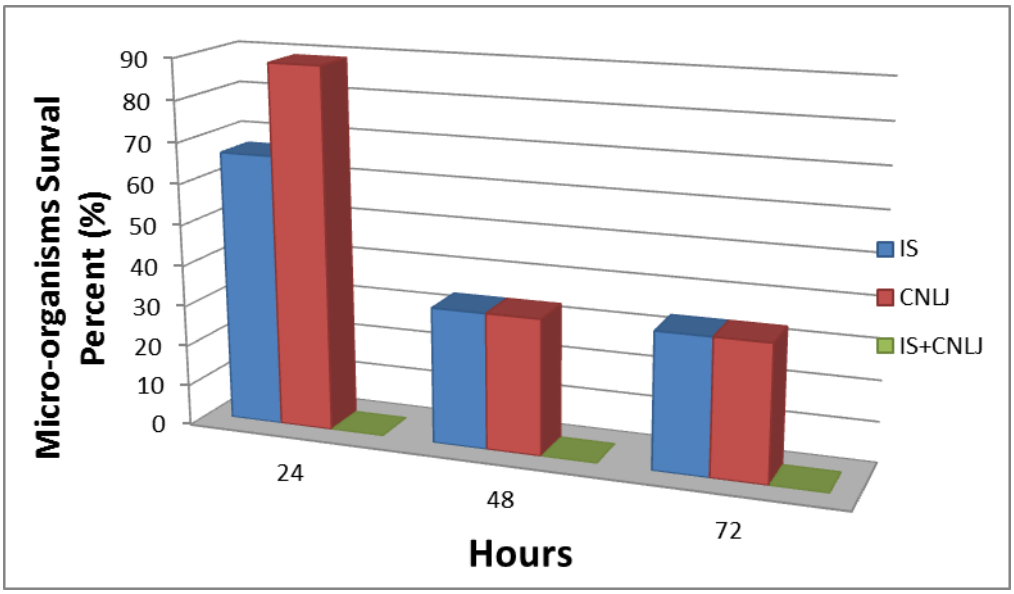


Fig. 2: Survival percentages in different materials

Moretro *et al*., (2009) found that nine disinfectants commonly used in the food industry and efficient against planktonic cells, showed a bactericidal effect that varied considerably for biofilm grown cells with products containing 70% ethanol being most effect.

Other studies similarly indicated that compared to planktonic cells, biofilm cells of *Salmonella* and *proteus* spp. were more resistant to trisodium phosphate, chlorine, and iodine (Scher *et al*., 2005; Joseph *et al*., 2001).

Although of Langsrud *et al*., (2003) openions about pseudomonas spp. resistance mechanisms against antimicrobial component commonly used in disinfectant such quaternary ammonium compound, this study was able to control on pseudomonas spp. growth by using a natural materials and the results showed no growth of this microorganisms during the three periods of treatment.

The failure of *Candida* spp., *Staphylococcus* spp., and *Streptococcus pneumonia* to be treated by (CNLJ) and (IS) separately for 24, 48 and 72 hours as it illustrated in table 1 may be due to many reasons like the penetration mechanisms of these materials because of thick layers of extrapolysaccharide which surrounded these microbial cells and prevent them from any disinfectant and if we supposed that these materials were penetrated the layers of biofilm, the microorganisms may be adapted to survive in these concentrated treatment materials.

Mixed *Candida-Staphylococcus* biofilms are similarly resistance to some of antimicrobial agents like fluconazole and there is evidence that the bacteria can enhance Candida resistance (Adam *et al*., 2002).

Gilbert *et al*., in (2002) have another idea about the mechanisms of biofilm resistance to antimicrobial agents when they said that these mechanisms are not fully understood, there is one long-standing hypothesis for the resistance of bacterial biofilms is that the matrix material restricts drug penetration by forming a reaction -diffusion barrier, and that only the surface layers of a biofilm are exposed to a lethal dose of antimicrobial agents, the extent to which the matrix acts as a barrier to drug diffusion would depend on the chemical nature of both the antimicrobial agent and matrix material (Shigeta *et al*., 1997).

The heterogenous nature of biofilm that consist of cells representing a wide variety of different metabolic states allows cells to survive a metabolically direct attack. (Costerton *et al*., 1999).

CONCLUSION

Locally, for the first time; combination of Iodized salt and natural lemon juice were found to have exceptionally cidal effect on the one month old biofilm forming microorganisms harbored on fresh fish cutting board during a period of 24 hours.

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