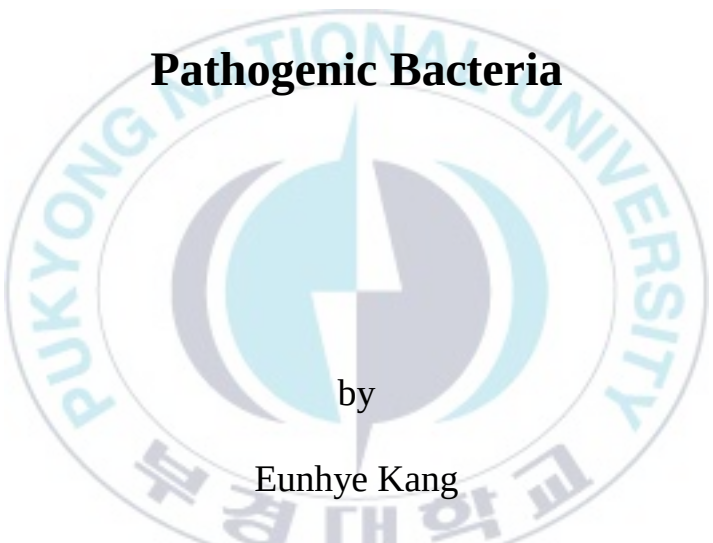
Thesis for the Degree of Master of Engineering

Antibiofilm Effects of Oligo-chitosan Compounds against Biofilm-forming Pathogenic Bacteria



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(바이오 필름을 형성하는 병원성균에 대한 올리고 키토산의 바이오 필름 생성 저해 효과)

Advisor: Prof. Young-Mog Kim

by

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List of Abbreviation

ATCC	**American Type Culure Collection**
BEC	**Biofilm eradication concentraion**
BIC	**Biofilm inhibitory concentration**
EPS	**Extracellular poylmeric substace**
KCCM	**Korean Culture Center of Microorganisms**
KCTC	**Korean Collection for Type Cultures**
MBC	**Minimum bactericidal concentration**
MIC	**Minimum inhibitory concentration**
TSA	**Tryptic soy agar**
TSB	**Tryptic soy broth**
MHA	**Mueller-Hinton agar**
MHB	**Mueller-Hinton broth**

바이오 필름을 형성하는 병원성 균에 대한 올리고 키토산의 바이오 필름 생성 저해 효과

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요 약

바이오 필름 (Biofilm)이란, 미생물들이 표면에 세포들이 서로 부착된 것을 말하며 ‘생물 막(生物膜)’ 이라고도 한다. 바이오 필름은 식품, 질병(식중독)뿐만 아니라 정수기, 상하수도, 산업용 배관, 공기 정화시설, 생활용품 등 모든 종류의 인공적인 시설물 표면에 미생물들이 부착하면서 형성된다. 특히, 인공장기 같은 의, 치과 의료기구, 각종 삽입 보형물 (implant)등 에서 미생물들은 활발히 바이오 필름을 형성하고 그로 인해 우리 몸에 질병을 발생시키게 된다. 그 뿐만 아니라 세탁기, 에어컨, 가습기를 포함한 생활가전 그리고 배수구 등 우리의 일상생활에서 넓고 다양하게 심각한 문제를

일으키고 있다.

바이오 필름은 여러 단계를 통해 복잡한 과정으로 형성된다. 바이오 필름이 생성되는 과정 중 박테리아들은 자기 생장에 있어 불리한 환경에 처할 경우, 환경에 적응하기 위해 quorum sensing을 이용하여 여러 가지 신호 물질을 생성하고 그 주위에 세포 밖 고분자 물질인 extracellular polymeric substance (EPS)를 생산한다. EPS는 고분자 복합체로써 세포 외부의 유전자, 단백질, 다당류로 이루어져있다. 이렇게 형성된 EPS는 바이오 필름의 3차원구조와 전반적인 골격을 구성하며 미생물 집단을 더욱 단단하고 끈끈하게 한다. EPS에서 자라는 미생물 균체들은 생리학적으로 액체 배양액에 떠다니거나 헤엄치는 플랑크톤의 단일 세포와는 구별된다.

바이오 필름의 문제점은 미생물 막(biofilm) 내의 세균들이 부유 상태의 세균(planktonic bacterial cells)보다 항생물질에 대한 내성이, 적게는 10배에서, 많게는 1,000배 이상 높아져 기존에 알려진 항생제로는 치료하기가 어렵다는 것이다. 생체 이외에서도 바이오 필름은 여러 가지 항생제나 살균제로부터 더 큰 저항성을 가지며 끈끈한 밀착력 때문에 살균과정에서 제거되지 않는다. 이로 인해 다른 식품과 직・간접적인 접촉으로 2차 오염을 야기하며 공중위생에

심각한 문제를 초래할 수도 있다. 또한 병원성 균에 의한 교차오염뿐만 아니라 부패 균에 의한 오염으로 식품에 심각한 영향을 줄 수 있기 때문에 바이오 필름의 제거가 반드시 필요하다.

바이오 필름을 잘 생성하는 대표적인 병원성 균들은 *Pseudomonas aeruginosa, Listeria monocytogenes, Staphylococcus aureus*가 있다. 이 병원성 균들을 대상으로 바이오 필름을 저해하기 위한 많은 연구가 진행 중이다.

본 연구에서는 항균, 항산화, 항염증 효과를 포함한 생리활성 등의 효과가 있다고 보고된 천연 해양물질인 chitosan과 이를 저 분자화 시킨 분자량이 다른 oligo-chitosan을 이용하여, 바이오 필름을 형성하는 병원성 균인 *P. aeruginosa, L. monocytogenes, S. aureus*를 대상으로 바이오 필름의 생성 저해 및 억제 효과를 측정하였다. 먼저 일반 부유균에 대한 항균 활성을 측정하기 위해, minimum inhibitory concentration (MIC) 와 minimum bactericidal concentration (MBC) assay를 이용하였다. 그 결과는 전반적으로 대조구인 chitosan보다 저 분자화시킨 분자량 1-3 kDa 인 oligo-chitosan이 뛰어난 활성을 나타내었다. 전체적으로는 oligo-chitosan의 분자량에 따라 유사한 항균 효과를 나타내었지만 oligo-chitosan분자량이 커질수록 효과는

감소하였다. 특히 실험에 이용된 식중독 균 중 *L. monocytogenes*가 가장 우수한 항균 활성을 나타내었고, 그 다음으로 *P. aeruginosa*와 *S. aureus*가 항균활성을 보였다.

Chitosan과 oligo-chitosan의 antibiofilm 활성 측정 실험은 병원성 균들에 의해 형성된 바이오 필름에 대한 chitosan과 oligo-chitosan의 바이오 필름 저해 효과를 파악하기 위한 실험이다. 실험방법은 biofilm inhibitory concentration (BIC)와 biofilm eradication concentration (BEC) assay를 진행하였고, 균의 종류와 물질에 따라서 항균 활성과 그 경향이 유사하였다. 또한 형성된 바이오 필름을 저해하는데 요구되는 Chitosan과 oligo-chitosan의 농도는 일반 부유균을 저해하는데 요구되는 값보다 최소 2배에서 최대 16배 높은 값을 나타내었다. 다음으로 chitosan과 oligo-chitosan이 바이오 필름 형성을 얼만큼 억제하는지 파악하기 위해 MIC의 농도 보다 3단계까지 낮은 sub-inhibitory concentration(sub MIC) 에서 (0.5 MIC, 0.25 MIC, 0.125 MIC) 형성된 바이오 필름을 염색하는 safranin stain assay를 진행하였다. Sub-MIC 농도(0.5 MIC, 0.25 MIC, 0.125 MIC)에서 safranin으로 염색된 바이오 필름은 chitosan과 oligo-chitosan이 바이오 필름 형성을 얼마나 효과적으로 억제하였는지 알 수 있다.

Safranin stain assay에서 TSB배지를 사용했을 때 샘플농도가 적어질수록 바이오 필름형성이 저해되어야 하는데 반대의 현상이 나타나는 문제점이 있었다. 이러한 이유는 chitosan과 배지의 어떠한 성분이 결합반응을 하면서 나타나는 현상으로 추정되고 또한 chitosan농도가 희석되면서 배지의 ph변화로 인해 chitosan가루가 well plate바닥에 가라앉는 현상이 나타난 것으로 추정된다. 이러한 문제점들을 개선하고자 MHB배지를 썼을 때 chitosan이 가라앉는 현상이 적게 나타났으며 MIC값도 더 효과적으로 나타났다. TSB배지를 MHB배지로 바꿔서 가장 우수한 항균 활성을 보인 *L. monocytogenes*에 대해 초점을 맞추어 safranin stain assay을 진행하였다.

종합적으로 병원성 균들의 일반 부유균 보다 바이오 필름이 가지는 항생물질에 대하여 증가된 저항성을 알 수 있었다. 이에 대한 원인은 균의 종류마다 각기 다른 메커니즘으로 바이오 필름의 형성이 이루어지기 때문이며 또한 항생물질에 대한 반응과 바이오 필름을 형성하는 구성 성분 및 구조가 다르기 때문으로 추정된다.

본 연구의 결과를 통해 oligo-chitosan이 항균, 항염증, 항암 활성뿐만 아니라 *L. monocytogenes* 바이오 필름에 대한 뛰어난 항균활성을 나타내는 것으로 판단된다. 향후, 항균 활성을 가지는 천연

유래 기능성 물질로서 올리고 키토산이 바이오 필름을 생성하는 병원성 균 제어에 유용하게 이용 되어 질 것으로 생각된다.

Introduction

Biofilms consist of groups of bacteria attached to surfaces and encased in a hydrated polymeric matrix (Banin et al., 2005). The ability of many pathogenic bacteria to adhere to surfaces and to form biofilms has major implications in a variety of industries including the food industry, where biofilms create a continuous source of contamination. The formation of a pathogenic bacteria biofilm is determined not only by the properties of the attachment surface, but also by the characteristics of the bacterial cell and by environmental factors (Van Houdt and Michiels, 2010). Biofilm is formed by attaching microorganisms to the surface of all kind of artificial facilities such as water purifier, water supply and sewerage, industrial piping, air purification facility, daily necessities as well as food illness (food poisoning). (Donlan, 2011)

Especially, in artificial organs, dental medical apparatuses and various implants, microorganisms actively form a biofilm, which causes diseases in our body. Not only that, but also household appliances including washing

machine, air conditioner, humidifier, and drain are causing serious problems in our daily life (Rayner et al., 2004).

The problem with biofilms is that bacteria in biofilms are very resistant to antibiotics 10 to 1,000 times more resistant than suspended (Rasmussen et al., 2006). It is believed that the bacterial traits are changed to inactivate antibiotics by extracellular polysaccharides or oxygen, or to slow the rate of growth due to biofilm formation and to obtain resistance (Wolcott and Ehrlich, 2008). In other words, biofilms with greater resistance from antibiotics and fungicides are not well removed during sterilization, so they cause secondary contamination through direct contact or indirect contact with other foods and may cause serious problems in public health.

Food surfaces are a good basis for the formation of biofilms. In the food industry, sanitary management is carried out through strict washing and disinfection procedures. The initial plankton cells and biofilm that have just been formed can be removed, but already well-developed biofilm is difficult to remove. They tend to settle in places that are difficult to access or have irregular surfaces, especially in areas that are difficult to clean. Bacterial cell migration from biofilm to food becomes a food safety and quality risk even after sanitizing. (Shi et al., 2009 ; Orgaz et al., 2011)

Pseudomonas aeruginosa, Listeria monocytogenes and *Staphylococcus aureus* are the most common pathogenic bacteria producing biofilms in food

processing plants (Deza et al., 2005 ; Poulsen 1999). *P. aeruginosa* biofilms cause persistent infections in individuals with underlying health problems and it is an opportunistic human pathogen causing respiratory tract infections in cystic fibrosis and skin infection in burn patients, as well as in other immunocompromised individuals (Banin et al., 2005 ; Zimmer et al., 2014). Biofilm formed by *L. monocytogenes* also causes a serious risk in food processing because they can persist for long periods of time in the food processing environment and thus represent a source of recurrent contamination (Kim, 2017; Møretrø and Langsrud, 2003). With the emergence of multidrug resistant and biofilm forming foodborne bacteria, researchers now constantly look for novel natural antimicrobial alternatives to treat and cure pathogenic microbial infections (Suleria et al. 2015). In this attempt, various natural products have been previously tested for antimicrobial and anti-biofilm potentials. Studies have been conducted to control pathogenic bacteria using plant natural products such as essential oils (Hammer et al., 1999 ; Oussalah et al., 2007), fatty acids (Ouattara et al., 1997), phenolics compounds (Puupponen-Pimiä et al., 2001), nano materials (Li et al., 2008) and marine natural products (Donia et al., 2003). Recently marine natural products received much recent attention due to its unmatchable chemical diversity. Marine natural products are reported to

offer unique chemical scaffolds that can be effectively utilized for the discovery of novel antimicrobials (Montesar et al., 2011).

Chitosan, a polysaccharide derived from partial deacetylation of chitin, is a linear polysaccharide composed of randomly distributed β-(1→4) linked D-glucosamine and N-acetyl-D-glucosamine. It has various biological activities including antitumor, antioxidant, anti-inflammatory, and enzyme inhibitory, drug delivery effect (Hamman, 2010). There have been reports on the antimicrobial potential of chitosan and its derivatives. Much of the research on the bactericidal effect of chitosan and oligo-chitosan compounds has been made on planktonic microorganisms (Fernandes et al., 2008 ; Carlson et al., 2007 ; van der Mei et al., 2007). The antimicrobial action mechanism of chitosan was thought to be due to its charge and interaction with the cell wall and cell membrane components (Liu et al., 2004).

However, the reports on the anti-biofilm and biofilm prevention effects of chitosan and its derivatives against important pathogenic bacteria are limited. For these reasons, it was evaluated the antibacterial and the antibiofilm potential of chitosan and oligo-chitosan compounds. Therefore, purpose in the present study was to evaluate the activity of different concentrations of native and degraded chitosan against mature biofilms of three bacterial species frequently associated with foodborne diseases: *P. aeruginosa, L. monocytogenes* and *S. aureus*. These results of the present

study suggested that chitosan and oligo-chitosan can be useful biomaterials with potential antimicrobial and anti-biofilm activity against food bone and human pathogenic bacteria. It is expected to be used as an important natural antimicrobial functional material with multiple health benefits to be used in healthcare and biomedical fields.

Materials and Methods

1. Materials

Chitosan (MW >250 kDa and 80% degree of deacetylation) prepared from crab shell chitin was purchased from by Kitto Life Co. (Seoul, Korea) and oligo-chitosans (MW 1-3kDa, 3-5kDa, 5-10kDa and 80% degree of deacetylation) were prepared according to the method previously reported by Park et al. (2004). All other chemicals and reagents used in this study were of analytical grade and commercially available .

Table1. List of Chitosan and Oligo-chitosan compounds used in this study

Compounds		Sources
Oligo-chitosan	1-3 kDa	Kitto Life Co. (Seoul, Korea)
	3-5 kDa	Dynesoze Co.
	5-10 kDa	Kitto Life Co. (Seoul, Korea)
Chitosan	250 kDa	Kitto Life Co. (Seoul, Korea)

2. Bacterial strains and culture conditions

The following microbial strains were purchased from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea). *P. aeruginosa* KCCM 11321, a reference bacterial strain was purchased from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea) to assure reliability of research results. *L. monocytogenes* standard strains KCTC 3569 and *Staphylococcus aureus* KCTC 1916 were purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). Other clinical strains *P. aeruginosa* and *L. monocytogenes* were obtained from the Gyeoingsang National University hospital. These strains were grown aerobically in tryptic soy broth (TSB; Difco Labotatory Inc., Detroit, MI, USA) and Mueller–Hinton broth (MHB; Difco Inc.), respectively at 37℃ and were subsequently used in the antimicrobial and biofilm assays.

Table 2. List of bacterial strains used in this study

Strains	Source
Pseudomonas aeruginosa **KCCM 11321**	**Korean Culture Center of Microorganisms (KCCM; Seoul, Korea)**
Listeria monocytogenes **KCTC 3569**	**Korean Collection for Type Cultures (KCTC; Daejeon, Korea)**
Staphylococcus aureus **KCTC 1916**	
P. aeruginosa **isolate 1842**	**Gyeongsang National University Hospital**
P. aeruginosa **isolate 4068**	
P. aeruginosa **isolate 4135**	
P. aeruginosa **isolate 366**	
P. aeruginosa **isolate 4562**	
P. aeruginosa **isolate 4561**	
L. monocytogenes **isolate 2148**	
L. monocytogenes **isolate 2868**	
L. monocytogenes **isolate 2637**	

3. A quantitative antibacterial assay against planktonic bacterial cells

Minimum inhibitory concentration (MIC) is the method of evaluating the antimicrobial activity quantitatively. It is defined as the lowest concentration of antimicrobial agents which will inhibit the visible growth of a microorganism after 20-24 h of incubation (Grierson and Afolayan, 1999). The experiment procedures were followed by the guideline of Clinical and Laboratory Standards Institute (CLSI, 2012). Minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium (Amyes et al., 1996). It can be determined from broth dilution minimum inhibitory concentration (MIC) tests by sub culturing to agar plates that do not contain the test agent. MBC was defined as the highest dilution showing ≥99.9% kill after 24 h of incubation (Saginur et al., 2006). The MBC is complementary to the MIC; whereas the MIC test demonstrates the lowest level of antimicrobial agent that inhibits growth, the MBC demonstrates the lowest level of antimicrobial agent that results in microbial death.

4. A quantitative assay for antibiofilm activity

Biofilms of all strains were formed on bottom of microtiter plates. The planktonic bacteria were removed after incubation for 24 h at 37°C. The wells were washed two times with phosphate buffer saline (PBS; 0.1 M, pH 7.4) and filled with 200 mL two fold dilutions of the chitosan phytochemical compounds. The plates were incubated for 24 h at 37°C. The OD490 was measured two times at 0 h and after incubation for 24 h. The biofilm inhibitory concentration (BIC) values were determined as the lowest concentration at which no increase in optical density compared with the 0 h OD490. Biofilms in the bottom of plate wells were scarified by a loop and spread over the surface of tryptic soy agar (TSA; Difco Inc.) plates and Mueller-Hinton agar (MHA; Difco Inc.), then incubated for 48 h at 37°C. The biofilm eradication concentration (BEC) values were determined as the lowest concentration at which no bacteria were grown on the TSA plates and MHA plates.

5. Safranin staining assay for determining biofilm formation

In this experiment, only the *Listeria* bacterium, which had good biofilm inhibitory effect, was tested. A 24 well of microtiter plates was used in place of the 96 well of microtiter plates in order to more clearly see the inhibitory effect of the biofilm. When TSB medium was used, chitosan bound to some of the media components and resulted in the precipitation on the surface of the polystyrene plate (Raafat et al., 2008). Further precipitated chitosan consisted of aggregated bacterial cells that are metabolically active. This gives rise to confusion that the cells arise form biofilm or aggregated cells due to chitosan. Further these cells are found to be metabolically active and resulted in error in estimating biofilm formation. Therefore, the experiment of changing the TSB medium to MHB medium showed that the problem of chitosan appeared in TSB was less.

The effect of different concentrations of chitosan and oligo-chitosan compounds on biofilm formation was investigated on microtiter plates (Cramton et al., 1999). All strains were grown for 24 h in 10 mL MHB with 1% glucose, diluted in growth medium to 5×10^5 CFU/mL and 1 mL was dispensed into each well of microtiter plates in the presence of 1 mL sub-inhibitory concentrations (sub-MIC) of chitosan-phytochemical compounds

(0.5, 0.25 and 0.125 MIC) and 1 mL medium (control). After incubation for 24 h at 37°C, each well was washed twice with PBS. Next dried, stained with 0.1% safranin for 1 min and washed with water. The stained biofilms were resuspended in 1 mL PBS and the cell suspended solution was measured at OD490 using an ELISA reader (GENios® microplate reader; Tecan Austria GmbH; Grödig, Austria).

6. Data interpretation

For the data interpretation of MIC values, Geometric mean values of MIC, MBC, BIC and BEC values are represented. The geometric mean (G-mean) values which are transformed data derived by using logarithms to generate a normal distribution was used (Bland et al. 1996). G-mean values were compared using two tailed t-tests using the graph pad quick calc. http://www.graphpad.com/quickcalcs

Results and Discussion

1. Antibacterial efficacy of chitosan and oligo-chitosan compounds on planktonic bacterial cells

In this study, it was focused on the antibacterial effect of chitosan against biofilm forming pathogenic bacteria. Typically, strains of *P. aeruginosa*, *L. monocytogenes* and *S. aureus* were used. The results of MIC and MBC values of chitosan and oligo-chitosan against pathogenic bacteria in TSB and MHB medium are represented in Tables 3 and 5, and Table 4 and 6, respectively. The results of the MIC test in TSB medium against all the test pathogens (*P. aeruginosa*, n=6 and *L. monocytogenes*, n=4) showed that the oligo-chitosan (1-3 kDa) exhibited profound antimicrobial effect compared to other chitosan molecules. Oligo-chitosan (1-3 kDa) exhibited an MIC range of 32-512 μg/mL with geometric mean (G-mean) of 147.03 μg/mL against all strains which is found to be lower than the 250 kDa chitosan which exhibited MIC range of 128-1,024 (G-mean, 238.8 μg/ mL). All other forms of chitosan exhibited significantly higher MIC values compared to oligo-chitosan (1-3 kDa). Earlier, the MIC value of chitosan against *L.*

monocytogenes was reported from 150 to 800 µg/mL (Goy et al., 2009), which was higher MICs than the results in this paper. Among the bacteria, *L. monocytogenes* were found to be susceptible for oligo-chitosan treatment. The results of the MBC showed overall higher value over MIC values for all chitosan samples against the test bacteria. Especially, oligo-chitosan (1-3 kDa) exhibited the lowest MBC (G-mean, 445.72 µg/mL) which is comparatively lower than chitosan (G-mean 512 µg/mL). On the other hand other chitosan molecules, 3-5 and 5-10 kDa oligo-chitosan molecules had significantly higher MBC values. Further from the results, it is evident that the toxic action of oligo-chitosan (1-3 kDa) action is more evident towards *L. monocytogenes* strains compared to *P. aeruginosa* and *S. aureus*. It was also performed MIC and MBC experiments with different media like MHB, in order to determine the effect of media components on the antibacterial activity of chitosan and its derivatives. When experiments were performed in MHB medium, it was employed only selected bacterial with good potential to form biofilms for further experiment. When tested under MHB medium, the MIC and MBC results obtained for all the chitosan samples were found to be better than the MIC values observed for the samples under TSB condition (Table 4 and 6). One specific observation made in this study was that oligo chitosan (1-3 kDa) was found to exhibit better antimicrobial activity in terms of MIC (G-mean, 86.14 µg/ mL) and MBC (G-mean,

344.55 µg/ mL) which when compared with 250 kDa chitosan (MIC, G-mean, 172.28 µg/mL; MBC, G-mean, 463.73 µg/mL) was significantly reduced. More information on the MIC and MBC values of other chitosan derivatives against test pathogens in MHB medium can be found in the Table 4 and 6.

Earlier the effect of media components on the antibacterial effect of chitosan was reported which suggested that MHB was suitable media where good MIC and MBC values were obtained for chitosan samples against test bacteria (Raafat et al., 2008). The better antimicrobial activities observed in case of MHB media for all the chitosan samples, especially oligo-chitosan was speculated to be due to its molecular weight and charge characteristics which is expected not to form complexes with the medium components (Raafat et al., 2008). Hence the sample is readily available to come in contact with the bacterial cells. As oligo-chitosan has smaller molecular weight (1-3 kDa) which is thought to better penetrate the cell wall structure in Gram-positive bacteria like *L. monocytogenes* and be able to bind and interact with the cell membrane (Orgaz et al., 2011). Over all from the present study it is suggested that lower molecular weight chitosan is tend to exhibit better antimicrobial activity irrespective of the growth media used for the antimicrobial testing.

Table 3. Minimum inhibitory concentrations (MIC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in tryptic soy broth

Strains	MIC(μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes KCTC 3569	128	256	512	128
L. monocytogenes isolate 2148	64	512	512	128
L. monocytogenes isolate 2868	128	512	1,024	128
L. monocytogenes isolate 2637	32	512	1,024	128
P. aeruginosa KCCM 11321	128	256	>1,024	128
P. aeruginosa isolate 1842	512	1,024	>1,024	1,024
P. aeruginosa isolate 4068	128	512	>1,024	256
P. aeruginosa isolate 4135	256	512	>1,024	256
P. aeruginosa isolate 366	128	512	1,024	256
S. aureus KCTC 1916	512	1,024	>1,024	1,024
G-mean	247.03	649.34	891.44	238.85

Table 4. Minimum inhibitory concentrations (MIC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in Mueller Hinton Broth

Strains	MIC (μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes KCTC 3569	32	128	512	128
L. monocytogenes isolate 2148	128	256	512	128
L. monocytogenes isolate 2868	64	256	512	128
L. monocytogenes isolate 2637	64	256	512	128
P. aeruginosa KCCM 11321	128	256	512	512
P. aeruginosa isolate 4135	128	512	1,024	128
P. aeruginosa isolate 366	128	512	512	256
G-mean	86.14	282.65	565.30	172.28

Table 5. Minimum bactericidal concentrations (MBC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in tryptic soy broth

Strains	MBC (μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes KCTC 3569	256	512	>1,024	256
L. monocytogenes isolate 2148	256	1,024	>1,024	256
L. monocytogenes isolate 2868	128	1,024	>1,024	256
L. monocytogenes isolate 2637	128	512	>1,024	256
P. aeruginosa KCCM 11321	256	512	1,024	256
P. aeruginosa isolate 1842	1,024	>1,024	>1,024	1,024
P. aeruginosa isolate 4068	1,024	1,024	>1,024	1,024
P. aeruginosa isolate 4135	1,024	1,024	>2,048	1,024
P. aeruginosa isolate 366	1,024	1,024	>2,048	1,024
S. aureus KCTC 1916	>1,024	1,024	>2,048	>1,024
G-mean	445.72	831.75	1,260.69	512

Table 6. Minimum bactericidal concentrations (MBC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in Mueller Hinton Broth

Strains	MBC (μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes KCTC 3569	128	1,024	>1,024	256
L. monocytogenes isolate 2148	512	1,024	>1,024	1,024
L. monocytogenes isolate 2868	128	1,024	1,024	256
L. monocytogenes isolate 2637	128	1,024	1,024	128
P. aeruginosa KCCM 11321	1,024	1,024	1,024	1,024
P. aeruginosa isolate 4135	1,024	1,024	1,024	1,024
P. aeruginosa isolate 366	512	512	1,024	512
G-mean	344.55	927.46	1,024	463.73

2. Inhibitory effect of chitosan and oligo-chitosan compounds on preformed biofilms

The ability of chitosan samples to inhibit biofilm formation as well as to eradicate the mature biofilms was determined. From the BIC and BEC experimental results, it is suggested that oligo-chitosan exhibited the significant anti biofilm effect against the tested pathogens. The strains used in the present study possessed the capacity to produce biofilms with low to high potential on polystyrene surface (Kim et al., 2016). Hence the antibiofilm effect observed for chitosan and other derivatives in this study was found to be strain dependent. The results of the BIC and BEC experiments performed in TSB medium are summarized in Table 7 and 9. From the Tables it is evident that the concentration of chitosan required was 2-16 times higher than the concentration required inhibiting the planktonic cells. Further oligo-chitosan (1-3 kDa) with BIC range of 128-2,048 µg/ mL (G-mean, 675.59 µg/ mL) was found to be better in terms of BIC when compared to the chitosan 250 kDa (MIC range, 512-8,192 µg/mL; G-mean, 1,552.09 µg/mL). Further details on the BIC values of the chitosan samples can be found in Table 7. Similar reduction in the biofilm formation was also observed in the case of biofilms formed in MHB broth. From Table 8, it can be evident that oligo chitosan (1-3 kDa) with G-mean (565.29 µg/mL) and

chitosan (250 kDa) with G-mean (512 μg/mL) exhibited almost equivalent pattern in the BIC values. However, other chitosan samples possessed significantly higher BIC values compared to 1-3 kDa and 250 kDa chitosan samples. The improved chitosan ability to inhibit the biofilm formation in MHB and TSB comparable with oligo (1-3 kDa) chitosan was speculated to have different mode of inhibition. In our opinion, TSB medium contains many cosamino acids which upon fermentation by bacteria release short chain peptides which was suspected to interact with large molecular weight chitosan (250 kDa) and results in precipitation. However, the solubility of chitosan in MHB is more even and hence can prevent the surface adhesion of bacteria and resulting in better BIC values along with oligo-chitosan (1-3 kDa). Although other chitosan samples (3-5 and 5-10 kDa) consist lower molecular mass, this study suggested relatively higher BIC values compared to 1-3 kDa and 250 kDa. More information on the BIC values of various chitosan samples against different test bacteria can be found in Table 8. Although it is not very clear at this stage that why 3-5 kDa and 5-10 kDa oligo-chitosan samples exhibit higher BIC values, it is expected there can be selective channels in biofilms that allow specific MW chitosan molecule and hence destabilize the biofilms (Panwar et al., 2008). Whereas high MW (250 kDa) due to its flocculation can physically damage the cells and prevent

biofilm formation. However, a detailed investigation on the molecular weight based activity of chitosan against biofilm formation is thus necessary.

The effect of various chitosan samples on biofilm eradication was performed under TSB and MHB medium conditions. The BEC values of chitosan against the test bacteria were represented in Table. 9. From this table it is evident that oligo-chitosan (1-3 kDa) exhibited BEC range of 1,024-8,192 µg/mL (G-mean, 2,896.30 µg/mL) which was superior than chitosan (MIC range of 2,048-16,384 µg/mL; G-mean 10,085.54 µg/mL). All other chitosan samples have relatively higher BEC values than oligo-chitosan with 1-3 kDa. Similar trend in the BEC values of oligo-chitosan with 1-3 kDa was also evident when the experiments were performed in MHB media. In fact, lower BEC values for oligo-chitosan with 1-3 kDa was recorded MIC range of 2,048-4,096 µg/mL when compared with chitosan (250 kDa) of MIC range with 4,096-16,384 µg/mL.

Mature biofilms are more resistant to biocides and antibiotics due to the presence of exopolymeric matrix, extracellular-DNA and lipopeptides and wall teichoic acids (Flemming and Wingender 2010). The better BEC observed for oligo-chitosan with 1-3 kDa was speculated to be due to the better penetration and interaction of oligo-chitosan (1-3 kDa) with the exopolymeric matrix components of mature biofilms resulting in biofilm destabilization. Earlier, similar role for chitosan-phenolic conjugate against

the mature biofilms of *C. albicans* (Panwar et al., 2016) was reported to be due to exopolymeric matric destabilization.

Table 7. Biofilm inhibitory concentrations (BIC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in tryptic soy broth

Strains	BIC (μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes **KCTC 3569**	512	8,192	8,192	512
L. monocytogenes **isolate 2148**	1,024	4,096	4,096	1,024
L. monocytogenes **isolate 2868**	1,024	4,096	4,096	1,024
L. monocytogenes **isolate 2637**	128	2,048	2,048	512
P. aeruginosa **KCCM 11321**	512	4,096	4,096	512
P. aeruginosa **isolate 1842**	512	2,048	2,048	4,096
P. aeruginosa **isolate 4068**	1,024	4,096	4,096	8,192
P. aeruginosa **isolate 4135**	2,048	4,096	4,096	8,192
P. aeruginosa **isolate 366**	512	4,096	4,096	2,048
S. aureus **KCTC 1916**	1,024	4,096	4,096	1,024
G-mean	675.59	3,821.7	3,821.7	1,552.09

Table 8. Biofilm inhibitory concentrations (BIC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in Mueller Hinton Broth

Strains	BIC (μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes KCTC 3569	256	1,024	1,024	128
L. monocytogenes isolate 2148	256	256	2,048	128
L. monocytogenes isolate 2868	256	1,024	2,048	128
L. monocytogenes isolate 2637	128	512	2,048	128
P. aeruginosa KCCM 11321	2,048	4,096	8,192	4,096
P. aeruginosa isolate 4135	4,096	4,096	8,192	4,096
P. aeruginosa isolate 366	1,024	4,096	8,192	2,048
G-mean	565.29	1410.46	3,360.09	512

Table 9. Biofilm eradication concentrations (BEC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in tryptic soy broth

Strains	BEC (μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes KCTC 3569	4,096	16,384	16,384	16,384
L. monocytogenes isolate 2148	2,048	8,192	16,384	16,384
L. monocytogenes isolate 2868	2,048	8,192	16,384	16,384
L. monocytogenes isolate 2637	2,048	8,192	16,384	8,192
P. aeruginosa KCCM 11321	1,024	8,192	16,384	8,192
P. aeruginosa isolate 1842	2,048	4,096	16,384	8,192
P. aeruginosa isolate 4068	4,096	8,192	8,192	16,384
P. aeruginosa isolate 4135	8,192	8,192	16,384	16,384
P. aeruginosa isolate 366	8,192	8,192	16,384	8,192
S. aureus KCTC 1916	2,048	16,384	16,384	2,048
G-mean	2,896.30	8,779.97	15,286.81	10,085.54

Table 10. Biofilm eradication concentrations (BEC) of the chitosan and oligo-chitosan compounds against food pathogenic bacteria in Mueller Hinton Broth

Strains	BEC (μg/mL)			
	Oligo-chitosan (1 - 3 kDa)	Oligo-chitosan (3 - 5 kDa)	Oligo-chitosan (5 - 10 kDa)	Chitosan (250 kDa)
L. monocytogenes KCTC 3569	4,096	4,096	8,192	8,192
L. monocytogenes isolate 2148	4,096	4,096	8,192	8,192
L. monocytogenes isolate 2868	4,096	8,192	16,384	8,192
L. monocytogenes isolate 2637	2,048	4,096	8,192	8,192
P. aeruginosa KCCM 11321	4,096	8,192	16,384	16,384
P. aeruginosa isolate 4135	4,096	8,192	16,384	4,096
P. aeruginosa isolate 366	4,096	4,096	8,192	8,192
G-mean	3,709.84	5,512.81	11,025.61	8,192

3. Inhibitory effect of chitosan and oligo-chitosan compounds on biofilm formation

In order to study the effect of chitosan and oligo-chitosans on the biofilm adhesion on the polystyrene surface, it was employed a biofilm formation inhibition assay. Here it was evaluated the biofilm formation by the test pathogens in presence of sub-lethal concentrations of test agents using a biofilm staining agent safranin. The antibiofilm effect of chitosan and oligo-chitosan compounds was strain dependent and no constant pattern of inhibitory effect on biofilm formation was evident against all the tested strains. Many components such as polysaccharides, polyproteins and extracellular-DNA and other components are implicated in the biofilm formation (Flemming and Wingender 2010). As a result, each bacterium has its own distinctive feature in biofilm formation. For example, when *P. aeruginosa* biofilms are formed, important combinations require polysaccharides such as alginate, Pel and Psl, and biofilm structures are mushroom-like structures. (Ryder et al., 2007; Banin et al., 2006).

From our MIC, MBC, BIC and BEC experiments, it was found the chitosan and oligo chitosan has specific effect on the *L. monocytogenes*. Hence it was further selected *L. monocytogenes* for the biofilm formation assay in two different media namely TSB and MHB, respectively. As it used TSB

medium for the biofilm formation experiments, it encountered a large increase in the background OD of safranin compared to the untreated controls for the representative *L. monocytogenes* strains (Fig. 1). However, this problem was solved when it used MHB (Fig. 2-5). In fact, it was found less interferences when used MHB for the biofilm formation assays. As shown in the figures, the formation of biofilm is inhibited dose dependently at sub-MIC. Table 11 summarizes biofilm formation inhibitory potential of chitosan and oligo-chitosan for the representative *L. monocytogenes* strains.

The mechanism of antimicrobial activity of chitosan and oligo-chitosan against biofilm-forming bacteria is not known precisely. To further investigate the antimicrobial mechanism of oligo-chitosan compounds against these bacteria, it is necessary to investigate what genes are involved in the biofilm formation stage and how they inhibit the transcriptional regulation of genes involved in the destruction of protein expression. In addition, there is a difference in the biofilm formation depending on the external temperature and the growth state of the bacteria. Therefore, RNA isolation and real-time quantitative PCR (RT-qPCR) will be conducted focusing on *L. monocytogenes* which exhibited the most superb inhibitory effect in both antibacterial and antibiofilm. As listed in Table 12, *L. monocytogenes* genes critical for biofilm synthesis (*flaA, fliP, fliG, flgE, motA, motB, prfA, degU, mogR, dnaK, agrA, agrB, agrC*) were investigated

using RT-qPCR. In detail, *flaA*, *fliP*, *fliG*, *flgE*, *motA* and *motB* genes play a role for initial attachment of biofilm formation while *agrA*, *agrB* and *agrC* genes function as quorum sensing. *dnaK* gene acts for stress response, and *prfA*, *degU* and *mogR* genes do transcriptional regulation (Ollinger et al., 2009).

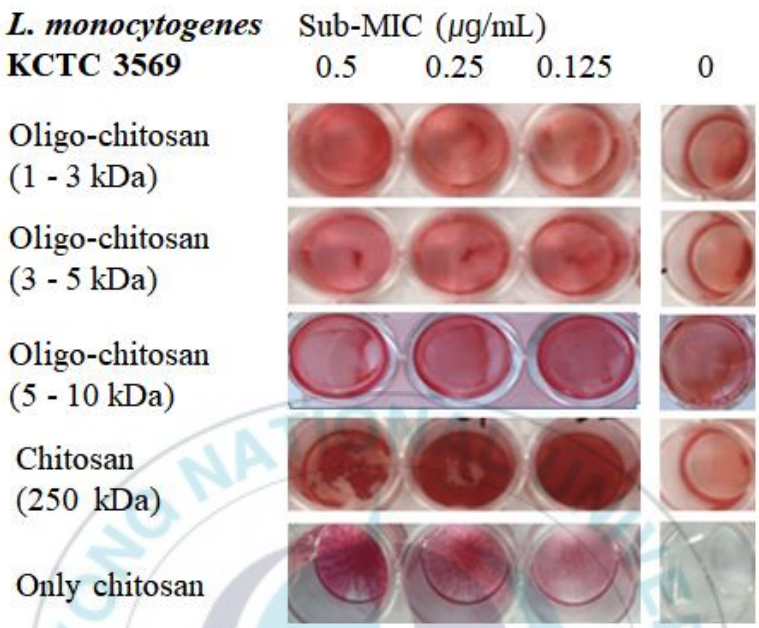

Fig. 1. Inhibitory effect of chitosan and oligo-chitosan compounds on biofilm formation against *Listeria monocytogenes* KCTC 3569 in tryptic soy broth

Table 11. Inhibitory effect of chitosan and oligo-chitosan compounds on biofilm formation in Mueller Hinton Broth

Strains	Samples	Biofilm formation value[a]		
		0.5 MIC	0.25 MIC	0.125MIC
L. monocytogenes KCTC 3569	Oligo-chitosan (1 - 3 kDa)	27.69605	75.21292	79.28327
	Oligo-chitosan (3 - 5 kDa)	38.66829	59.55093	56.27696
	Oligo-chitosan (5 - 10 kDa)	32.72262	57.69067	61.01406
	Chitosan (250 kDa)	44.86229	87.42396	94.06039
L. monocytogenes isolate 2148	Oligo-chitosan (1 - 3 kDa)	32.0052	54.77313	96.01561
	Oligo-chitosan (3 - 5 kDa)	46.31647	68.8242	93.67377
	Oligo-chitosan (5 - 10 kDa)	36.38688	65.01917	70.8138
	Chitosan (250 kDa)	64.01041	79.2324	100.9595
L. monocytogenes isolate 2868	Oligo-chitosan (1 - 3 kDa)	33.94395	39.5804	66.88586
	Oligo-chitosan (3 - 5 kDa)	39.07938	46.21888	99.07625
	Oligo-chitosan (5 - 10 kDa)	17.38389	59.65062	63.40009
	Chitosan (250 kDa)	68.38891	85.92453	117.4886
L. monocytogenes isolate 2637	Oligo-chitosan (1 - 3 kDa)	21.14057	54.81293	78.13295
	Oligo-chitosan (3 - 5 kDa)	32.89697	60.00308	84.02895
	Oligo-chitosan (5 - 10 kDa)	24.79761	57.43502	62.20707
	Chitosan (250 kDa)	85.3251	93.38903	115.1834

a.Biofilm formation values were calculated as: (mean OD490 treated well) / (mean OD490 control well) × 100.

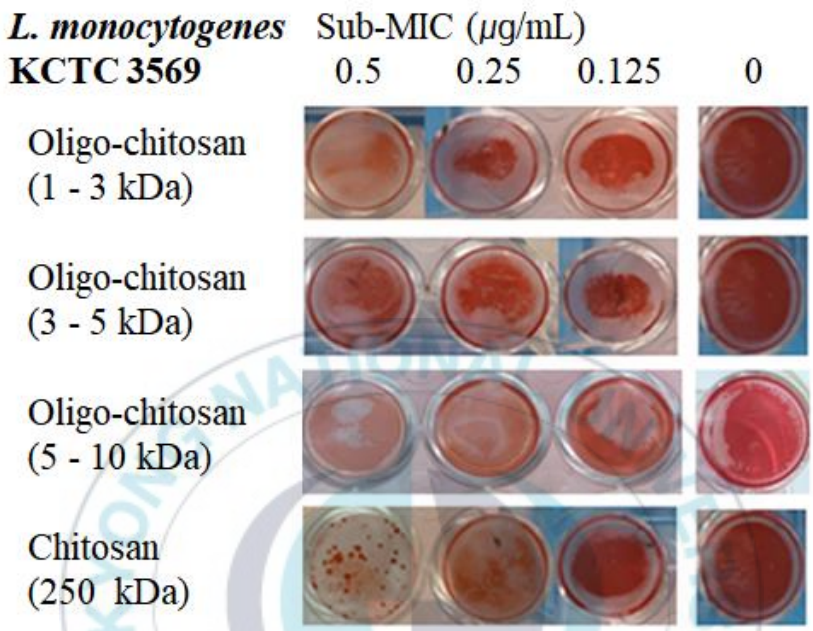

Fig. 2. Inhibitory effect of chitosan and oligo-chitosan compounds on biofilm formation against *Listeria monocytogenes* KCTC 3569 in Mueller Hinton Broth

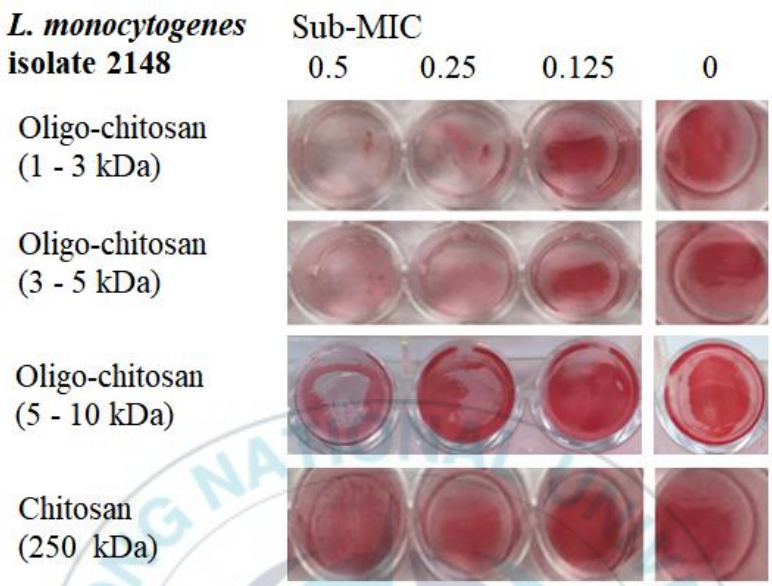

Fig. 3. Inhibitory effect of chitosan and oligo-chitosan compounds on biofilm formation against *Listeria monocytogenes* isolate 2148 in Mueller Hinton Broth

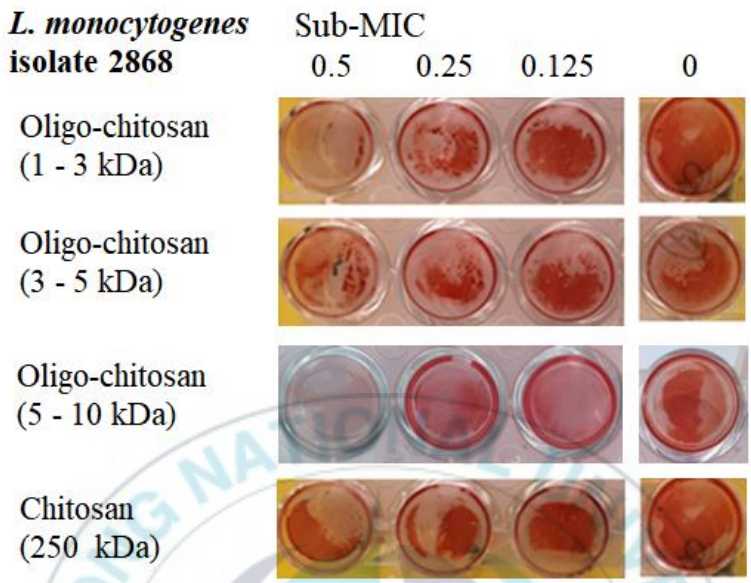

Fig. 4. Inhibitory effect of chitosan and oligo-chitosan compounds on biofilm formation against *Listeria monocytogenes* isolate 2868 in Mueller Hinton Broth

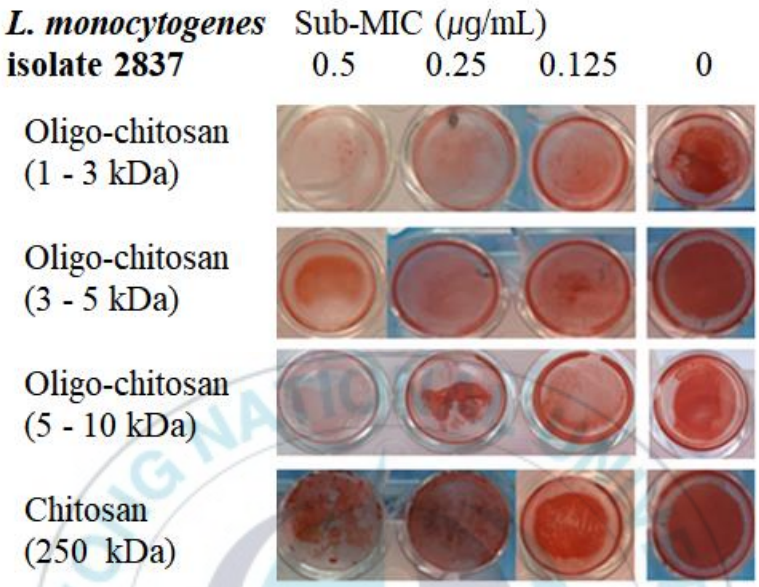

Fig. 5. Inhibitory effect of chitosan and oligo-chitosan compounds on biofilm formation against *Listeria monocytogenes* isolate 2837 in Mueller Hinton Broth

Table 12. Functions of *Listeria monocytogenes* genes associated with Biofilm forming

Gene	Function
flaA	Structural flagella protein
flip	Flagellar biosynthesis protein
fliG	Flagellar motor switch protein
flgE	Flagellar hook protein
motA	Flagellar motor protein
motB	Flagellar motor protein
prfA	Transcriptional regulator
degU	Transcriptional regulator/quorum sensing
mogR	Transcriptional regulator for motility
dnaK	Molecular chaperon involve in biofilm
agrA	Quorum sensing
agrB	Quorum sensing
agrC	Quorum sensing

Conclusion

With the emergence of multi-drug resistant, biofilm forming pathogenic microorganisms and acute shortage of novel antibiotics, there is an urgent need to research and invent novel antimicrobial agents to fight the dreadful pathogens forming biofilm. Hence this research was aimed to provide natural solutions to the existing antibiotic crisis. Here it was investigated the antimicrobial potential of chitosan and oligo-chitosan compounds against biofilm-forming and multi-drug resistant bacterial strains such as *P. aeruginosa, L. monocytogenes* and *S. aureus* by various standard antimicrobial techniques. In the current study it was evaluated the inhibitory efficacy of chitosan, oligo-chitosan compounds on biofilm was evaluated by MIC and MBC for planktonic bacterial cells and BIC and BEC for biofilm cells.

In general, the antimicrobial efficacy of chitosan and oligo-chitosans was strain and media dependent. It was observed a more reproducible and strong antimicrobial effect for chitosan and oligo-chitosan samples in the MHB medium rather than TSB medium. Further, chitosan and oligo-chitosan

compounds exhibited toxicity against *L. monocytogenes*, followed by *P. aeruginosa* and *S. aureus* in both antibacterial and antibiofilm activities. From the studies it was evident that among the samples low molecular weight oligo-chitosan (1-3 kDa) was very effective in terms of antimicrobial and antibiofilm effects against the test pathogens. A general trend in the reduction of geometric mean values of MIC, MBC, BIC, and BEC values was evident with oligo-chitosan (1-3 kDa) compared with 3-5, 5-10 and 250 kDa chitosan samples. Additionally, sub-lethal concentrations of oligo-chitosan samples (1-3 kDa) effectively prevented the biofilm formation by *L. monocytogenes* strains.

Considering the need for urgent development of biocompatible agents with multiple healths functional benefits as antimicrobials, oligo-chitosan samples of the present study are expected to useful biomaterials with high antimicrobial and antibiofilm potential and can be applied in food, healthcare and biomedical fields as effective antimicrobials. Although, this is a preliminary report on the antimicrobial potential oligo-chitosan, however, a thorough investigation on the mechanism of action of oligo-chitosan samples and its efficacy in *in vivo* animal models is expected to provide in depth understanding on the practical application of oligo-chitosan compounds as antimicrobials.

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