December 30, 2018

PharmacologyOnLine

Archives • 2018 • vol.3 • 150-160

ANTI-*LISTERIA* ACTIVITY OF NATURAL PRODUCTS SINGLE AND MIXED OBTAINED FROM NATIVE PLANTS OF ARGENTINA

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Abstract

A total of 66 samples of seafood were tested for the presence of *Listeria monocytogenes* and other *Listeria* species. *Listeria* spp. were present in 23% of the samples (15/66) of them, 10 isolates were characterized as *L. innocua* (66.67%), 2 L. monocytogenes (13.33%), 1 *L. grayi* (6.67%), 1 *L. seeligeri* (6.67%) and 1 *L. ivanovii* (6.67%). Of the total bacterial isolates, the 20% (3/15) showed ability to form biofilm, identified as 2 species of *L. innocua* and 1 *L. monocytogenes*.

The anti-*Listeria* activity of aqueous extracts of *A. caven, A. furcatispina, P. torquata* and *N. cataria* and their combinations was determined. The aqueous extract of *P. torquata* and *N. cataria* showed the highest inhibitory activity against strains of *Listeria* (2.5 mg/mL and 5 mg/mL respectively). The antibacterial activity was enhanced by the mixture of some extracts. The combinations of *A. caven* + A. *furcatispina* and *A. furcatispina + P. torquata* showed beneficial effect to inhibit the growth of Listeria. The fractional inhibitory concentration (FIC) index for this mixture indicated an additive effect (FIC = 0.75 and 0.56 respectively), while the remaining combinations showed indifferent effect. However, none of natural products, as well as all mixtures showed inhibitory activity in biofilm formation by *Listeria* at least, under the conditions assayed.

Keywords: *anti-Listeria activity, Acacia spp, biofilm, combinations of natural products, Nepeta cataria, Prosopis torquata*

Introduction

Since ancient times, plants are used as alternative medicine for the extensive properties they own. Plants have been a valuable source of natural products for maintaining human health. The antimicrobial properties of plants have been investigated by a number of researchers worldwide, especially in Latin America. In Argentina, a research tested 122 known plant species used for therapeutic treatments (1).

Currently, food safety remains a topic of great interest to public health, even if they have improved and modernized hygiene practices in refrigerators and food production techniques. It is estimated that in industrialized countries, about 30% of the population suffers every year, food borne illness. Therefore, there remains a need for new methods to reduce or eliminate food borne pathogens, possibly in combination with existing. In addition, the ability to form biofilms on different surfaces, contributes to the biofilm-producing bacteria are one of the major concerns for the food industry (2, 3, 4). In this sense, new strategies are designed to eradicate biofilms and are increasing the use of substances with natural active ingredients.

Listeria monocytogenes is responsible for producing listeriosis, a fatal disease it is most prevalent in immune-compromised individuals. The increase in numbers of immune-compromised individuals against a background of *Listeria* antibiotic resistance, limits listeriosis treatment options. This therefore calls for research into substitute treatments, of which, medicinal plants derived compounds offer a viable alternative.

The genus *Listeria* comprises fifteen species but only two are considered pathogenic (5). *L. ivanoii* is pathogenic for animals like cattle and small ruminants, while *L. monocytogenes* is pathogenic for both animals and humans (6, 7). *L. monocytogenes* is a gram-positive, intracellular facultative anaerobic bacillus, with optimum growth range of 30-37 °C but can grow at refrigerator temperatures. This species is cause listeriosis, which can produce gastroenteritis, septicemia, perinatal infections, fetal death, abortion, meningitis and meningoencephalitis in immunocompromised individuals (8, 9).

Contaminated foods such as raw vegetables, meats and ready to eat foods are the major source of pathogenic *Listeria*, such that the gastrointestinal tract is the bacteria's primary site of entry (10).

This disease occurs with a high mortality rate worldwide, ranging between 20% and 30%, reaching up to 80% in cases of neonatal infections (11). *L. monocytogenes* is widely distributed in the environment so that man is exposed to the bacteria in several ways. Approximately 99% of human listeriosis infections appear to be food borne (12). It has been isolated from various foods, especially those raw and ready-to-eat foods, cabbage, pasteurized milk, cheese, vegetables, raw fish and seafood (13).

Within virulence factors, it is their potential ability to produce biofilms, both biotic surfaces (plants, tissues) and abiotic (stainless steel, plastic) (14). This ability to adhere to the surfaces of industrial facilities leads to serious health problems and economic losses. The biofilm serves as a reservoir for continuous release of bacteria processed foods in contact with those surfaces, as it is found that adherent bacteria are more resistant to the processes of cleaning and sanitizing allowing survival for long periods of storage and difficult to eradicate (15). Currently, food safety remains a topic of great interest to public health, even if they have improved and modernized hygiene practices in refrigerators and food production techniques. It is estimated that in industrialized countries, about 30% of the population suffers every year, food borne illness. Therefore, there remains a need for new methods to reduce or eliminate food borne pathogens, possibly in combination with existing. In addition, the ability to form biofilms on different surfaces, contributes to these bacteria are one of the major concerns for the food industry (2, 3, 4). In fact, biofilms, densely packed communities of microbial cells growing in a living or inert surface and surrounded by a self-produced polymeric matrix, requires much higher doses of antibiotics (10–1000 times) for bacterial killing and can lead to chronic and persistent infections (16). This occurs because biofilm protects bacteria against several physicochemical aggressions, including ultraviolet light, heavy metals, acidity, modulation in hydration or salinity, and phagocytosis (17). In this sense, new strategies are designed to eradicate biofilms and are increasing the use of substances with natural active ingredients (18). Hence, more studies pertaining to

the use of plants as therapeutic agents for their application both in clinical medicine and in the food industry should be emphasized. Vegetable extracts from various plants native to Argentina, such as *Acacia caven, Acacia furcatispina, Prosopis torquata* and *Nepeta cataria* have been used by people to treat diseases as a form of folkloric medicine. The aims of this study were to investigate the ability of biofilm production of *Listeria* strains isolated from frozen raw seafood and to evaluate the anti-*Listeria* effect of the plants extracts mentioned before alone and combined and their effect on biofilm-grown.

Methods

Samples and isolates bacterial

Sixty six samples of seafood were analyzed. From frozen raw seafood such as are sold commercially, 10 g of each sample was weighed and incubated in Palcam broth 24 h at 37° (pre-enrichment) and then, subcultured in Palcam agar and incubated 48 h at 37°C. If growth is not detected in Palcam agar, isolating was retried from cold selective enrichment in Palcam broth for 7 days at 4°C. Suspicious colonies of *Listeria* spp were recognized for being small, black, and greenish, with depressed center and blackening of the medium. These colonies were inoculated in trypticase soy agar (TSA) and incubated 24 h at 37°C. Identification of *Listeria* spp was carried out using the standard biochemical tests including Gram staining, motility test fresh, catalase, hemolysis on horse blood agar and sugar fermentation (19). These bacterial isolates together with reference strains Collection Microbiology Laboratory strains were used in subsequent assays. Bacterial strains were maintained in Lauria Bertani broth (LB) containing 20% glycerol at -80°C until use.

Plants aqueous extracts

Aqueous extracts (decoctions) from aerial parts of *Acacia caven* var. caven DC (A), *Acacia furcatispina* (B) and *Prosopis torquata* Cav. Ex. Lag DC (C) were prepared as previously described Martinez et al, 2014 (20). Aqueous extract of *N. cataria* (D) previously obtained by Arce et al was also used in this study (21). They were sterilized by membrane filtration of 0.20 µm cellulose and stored at -20 °C until use. The 1:1 combinations of A+B, A+C, A+D, B+C, B+D and C+D were also assayed.

Antibacterial activity: Determination of minimal inhibitory concentration (MIC)

The MICs of extracts A, B, C and D were determined by microplate method (micro-well dilution) according to CLSI method (22), in tripticase soy broth (TSB) (Britania, Argentina) pH 7.2 supplemented with 0.01% (W/V) of 2,3,5-triphenyltetrazolium chloride as visual indicator of bacterial growth. A starting concentration of 40 mg/ml of each aqueous extract dissolved in 1% DMSO was serially diluted double fold in TSB to make different test concentrations of the compounds in the wells. The 96-well plates were prepared by dispensing into each well 95 µL of nutrient broth and 5 µL of the inoculum (106 CFU/mL). One hundred microlitres aliquot from the stock solutions of the extracts and their serial dilutions initially prepared were transferred into eight consecutive wells. The final volume in each well was 200 µL. Controls of nutrient broth, strains and extracts were also included in the experiment. The 1% DMSO was also tested for its possible anti-*Listeria* activities. The plates were incubated at 37°C for 24 h. A colour change (viewing with the naked eye) from colourless to red, indicated actively growing bacteria. MIC was defined as the lowest concentration of the extracts in the medium in which there was no visible growth. Assays were performed in duplicate and then replicated at least twice.

Determination of minimal bactericidal concentration (MBC)

The aqueous extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture on the surface of the TSA plates, in order to evaluate bacterial growth. MBC was interpreted as the minimum concentration of the antibacterial agent that prevented the growth of viable colonies on the tripticase soy agar after 24 h of aerobic incubation at 37° C. Assays were performed in duplicate and then replicated at least twice.

Determination of the antibacterial activity of combined extracts

The 1:1 combinations of A+B, A+C, A+D, B+C, B+D and C+D were assayed. In each initial combination, the concentration of each individual extract was 20 mg/mL. Then, serial two-fold dilutions were made in concentration ranges from 20 mg/mL to 0.625 mg/mL. The 96-well plates were prepared by dispensing into each well 95 µL of nutrient broth, 5

µL of the inocula and 100µL of extracts mixture and their serial dilutions initially prepared. The final volume in each well was 200 µL. Controls of nutrient broth, strains and extracts were also included in the experiment. The plates were incubated at 37°C for 24 h. Assays were performed in duplicate and then replicated at least twice. MIC values were determined for each of these combinations to establish any interaction effect. The MIC of mixture was compared with MICs of each extract separately to obtain the fractional inhibitory concentration (FIC) index (23). The FIC index of extract A (FICA) and extract B was calculated as the ratio of the MIC value of the extract in combination (A + B) over the MIC value of the extract alone (A or B). The overall FIC index (ΣFIC) was calculated as the summation of the FICA and the FICB. The interactions were interpreted as synergism when the ΣFIC index≤0.5, additive when 0.5<ΣFIC index≤1, and indifference when 1<ΣFIC index<4 whilst antagonism was defined as when the ΣFIC index is ≥4.

Quantitative determination of biofilm production

Biofilm production was carried out as described previously (24). Briefly, in each well 150 µL of TSB supplemented with 1% glucose and 50 µL of inoculums *Listeria* spp (108 CFU/mL) were inoculated into 96-well U bottom polystyrene microtiter plates and incubated at 37 ° C for 72 h. The wells were washed 3 times with phosphate buffered saline (PBS, pH 7.2), air dried and stained with 1% crystal violet solution for 15 min and then, fixed with methanol (200 µL) for 15 min. The unbonded dye was removed and washed with distilled water. The plate was allowed to dry air for 24 h. The content of the wells was resuspended with ethanol and was transferred to a flat bottom plate. The optical density (OD) the wells was measured at 550 nm using ELISA reader (Microplate Reader Benchmark, BIO-RAD). For classification of isolates according to their ability to form biofilms, a cut-off value was obtained (ODc =0.300). Values above or below this point determined producing or non-producing biofilm strains respectively.

Positive and negative controls strains were used as was a negative control of medium. Positive biofilm *S. aureus* strain 11F2, positive biofilm *L. monocytogenes* CLIP 74904 and negative biofilm *S. epidermidis* ATCC 12228 were included as controls. The tests were carried out in quadruplicate and were replicated at least twice, and the averages and standard deviations were calculated for all repetitions of the experiments.

Determination of anti-biofilm activity of extracts of plants and their combinations

The aqueous extracts of *A. caven* (A), *A. furcatispina* (B), *P. torquata* (C) and *N. cataria* (D) and the combinations (1:1) of A+B, A+C, A+D, B+C, B+D and C+D at their MIC value were tested for their ability to interfere with the biofilm production by 4 strains of *Listeria* and 4 strains of *S. aureus*. An aliquot of 50 µL of each bacterial suspension (108 CFU/mL), 50 µL of TSB supplemented with 1% glucose and 100 µL of extracts or mixture were placed into each well of 96-well plates. After 72h incubation at 37° C, the content was aspirated and the plates were washed with PBS, dried, fixed with methanol and stained with 1% crystal violet solution in water for 15 min. The content adhered was resuspended in 200 µL of ethanol and read in ELISA reader. OD550 ≥ 0.300 was considered producing biofilm. The tests were carried out in quadruplicate and were replicated at least twice, and the averages and standard deviations were calculated for all repetitions of the experiments.

Results and Discussion

Fifteen (15/66) isolates of *Listeria* were collected from seafood samples (23%), of them, 10 isolates were characterized as *L. innocua* (66.67%), 2 *L. monocytogenes* (13.33%), 1 *L. grayi* (6.67%), 1 *L. seeligeri* (6.67%) and 1 *L. ivanovii* (6.67%). All bacterial isolates were genetically confirmed at the Pasteur Institute Center *Listeria*, Francia.

The presence of *Listeria* species in raw frozen shellfish warns about the importance of proper cooking of these foods before ingestion and the feasibility of these organisms to generate cross contamination in seafood processing plant environments found higher percentages of *L. monocytogenes* (60%) and *L. innocua* (30%) (25). Conversely, Rozman et al, 2016, only found *L. innocua* (15%) and *L. monocytogenes* was not identified in any of the examined samples of fish and fishery products (26).

The microbiological risk for food consumption is one of the main ones reasons for concern for Public Health Organizations and the food sector. In this field, *L. monocytogenes*, is an important concern for the meat sector and, in particular, for productive

and product marketing companies meat ready for consumption (RFC). At present, there is a debate on the policies of regulation of this pathogen that apply in different countries of the world and that affect product marketing traditional meat. In the international guidelines of Codex Alimentarius and online with food security policy at community level (Regulation (EC) 2073/2005) or extra-community (Canada), different microbiological criteria are established based on two risk factors: the susceptibility of the population to which food is destined and the possibility that food favors or does not increase the pathogen during its useful life.

In the European Union (EU), a maximum of 100 cfu/g is established during the life of products not intended for the population of risk (children, elderly, pregnant women or immunodeprimised), regardless of whether the pathogen may or may not grow in RFC products. On the other hand, country legislation as the United States and Japan at the beginning were very restrictive, since established a policy of "zero tolerance" (absence in 25g) in front of this pathogen, demanding it both in RFC foods and in the production environment. However, these countries have expanded their regulations, using only the "zero tolerance" criteria for RFC products where *Listeria* is able to grow.

Our results, as well as numerous investigations, demonstrate the presence of *Listeria* species in fish, seafood or in sea food processing plants (27, 28, 29). These findings are indicators of the importance of standardized sanitation procedures to reduce risks regarding to this emerging pathogen of significant impact on public health.

Evaluation of anti-*Listeria* effect

The aqueous extracts of *A. caven, A. furcatispina, P. torquata, N. cataria* and their combinations showed anti-*Listeria* activity. The values of MIC varying from 20 to 2.5 mg/mL (Table 1). The aqueous extract of *P. torquata* and *N. cataria* showed the greatest inhibitory activity against strains of *Listeria* (2.5 mg/mL and 5 mg/mL respectively) and the least active was *A. furcatispina* (20 mg/ml). Aqueous and ethanolic extracts of both species of *Acacia* and *P. torquata* in previous studies (20) showed MIC values of 2 and 4 mg/mL against other Gram-positive bacteria (*Staphylococcus aureus*). In general, values of MIC were bacteriostatic. The 1% DMSO was found not to exhibit anti-*Listeria* activities *in-vitro*. Higher concentrations (one or two fold higher than the corresponding MICs values) of decoctions were needed to have bactericidal effect.

Previous phytochemical study of the A. caven, A. furcatispina and P. torquata revealed the presence of carbohydrates, tannins, flavonoids, saponins and alkaloids either in both aqueous and ethanol extracts or in any of them. TLC analysis revealed the presence of flavonoids: quercetin (Rf of 0.91) and sapogenines: oleanolic acid (Rf of 0.87) (20). Nepetalactone compounds mainly found in the essential oils of the Nepeta species are considered to be responsible for the pharmacological properties (30).

Recently, plant antimicrobials have been found to be synergistic enhancers in that though they may not have any antimicrobial properties alone, but when they are taken concurrently with standard drugs they enhance the effect of that drug (31, 32).

There are some generally accepted mechanisms of this interaction, including inhibition of protective enzymes, combination of membrane active agents, sequential inhibition of common biochemical pathways, and the use of membranotropic agents to enhance the diffusion of other antimicrobials (33).

Phytotherapy has many potentially significant advantages associated with the synergistic interactions like, increased efficiency, reduction of undesirable effects, increase in the stability or bioavailability of the free agents and obtaining an adequate therapeutic effect with relatively small doses, when compared with a synthetic medication (34).

Interactions between plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic this outcome). In our study, the combinations of A. caven + A. furcatispina and A. furcatispina + P. torquata showed beneficial effect for Listeria. The MIC data of the mixtures are presented in Table 2. The FIC index for this mixture indicated an additive effect ($0.5<\Sigma FIC\ index\leq 1$), while the remaining combinations showed indifferent effect (Table 2).

All the mixture tested showed bactericidal effect against *Listeria* strain at a maximum concentration of 1× MIC or 2× MIC value of the tested extract.

An additive interaction is the effect where the combined action is equivalent to the sum of the

activities of each drug when used alone (35). The other combinations tested showed no effect synergistic, or additive, or indifferent or antagonistic. The decrease in one or two dilution steps above the MIC values observed in this work can be considered only as partial synergism (36). The additive effect of *A. caven* + *A. furcatispina* and *A. furcatispina* + *P. torquata* observed herein, probably, suggests the therapeutic applicability of such extracts in combination therapy. However, it is hard to predict combined effects in vivo on the basis of the presented in vitro evidence alone. It must be studied in animal models to determine their efficacy in vivo, and to elucidate their mechanisms of action.

A. caven and *A. furcatispina* showed good inhibitory activity on the growth of *Listeria* species. The mixture or combination of both *Acacia* species had an additive effect on their bacteriostatic activity. In addition, these plants do not have toxicity. A previous study of our research group determined that these species are not genotoxic below 10 mg/mL concentration (37). Another species of the genus *Acacia*, *A. aroma*, was previously tested and also had a bacteriostatic/bactericidal effect and showed no cytotoxic and genotoxic activity (38). Therefore, the use of the mixture of both *Acacia* species represents a potential natural product for safe use in phytotherapy.

In contrast, *P. torquata* showed very good antibacterial activity both alone and combined with *A. furcatispina* but was genotoxic at concentrations of 0.2 mg/mL (37). Consequently, our next objective is to obtain greater bioactivity through chemical modifications that reduce toxicity of *Prosopis*.

Biofilm production

L. monocytogenes is able to form biofilm on several surfaces used in the food industry, representing a serious concern for food safety because it could serve as source of contamination. In fact, *L. monocytogenes* has been isolated from a wide range of processed foods (39, 40, 41) and also cooked food can be contaminated as the results of post-process contamination (42). Microtitre assay is an effective method to identify biofilm forming species. This method allowed to identify 3 *Listeria* isolates producing biofilm characterized as *L. innocua* (13%, 2/15) and *L. monocytogenes* (7%, 1/15) (Table 3, Fig. 1). The species *L. grayi, L. ivanovii* and *L. seeligeri* were no producer biofilm (OD<0.300).

Evaluation of anti-biofilm effect

Given the potential existence of biofilm-producing strains in food and / or food processing plants, it is a challenge to find substances with anti-biofilm activity. The plants and derived products offer a potential natural alternative. Some natural products have the ability to inhibit the formation of the biofilm formed as well as prevent the formation thereof (43). Conversely, other plants do not have this effect. Many of these agents have their activity tested in planktonic cultures. However, the activity on sessile forms of life, responsible for the gravest problems, is still understudied. Studies that evaluate antibiofilm activity should deal with the activity against consolidated biofilms and anti-adherent properties as a prophylactic measure against the formation of biofilm (44).

Sanchez et al, 2016 found inhibitory activity of biofilm against *E. coli* and *S. aureus* in a species of the genus Prosopis (45). Contrary, the results obtained in our work determined that all tested natural products single showed no inhibitory activity in biofilm formation by *Listeria*. None of the combinations tested showed inhibitory effect of biofilms at least under the conditions of the assay. Further research is required to find an effective way to clean the surfaces and effective sanitization methods to completely remove this bacterium.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CM Mattana wrote the manuscript. AM Mohamed carried out the experimental part and collaborated with the writing of the paper. CM Mattana and DA Cifuente participated in the design of the study and the critical revision of the manuscript and data content. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to acknowledge the National University of San Luis, Project 02-0218.

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Table 1. The MIC and FIC values of vegetable extracts for strains of *Listeria* tested.

Vegetable Extracts	A	B	C	D	A+B	A+C	A+D	B+C	B+D	C+D
MIC (mg/ml)	10	20	2.5	5.0	5.0	2.5	5.0	1.25	10	5.0
FIC					0.75	1.25	1.5	0.56	2.5	3.0

A: *A. caven*. **B**: *A. furcatispina*. **C**: *P. torquata*. **D**: *N. cataria*. **MIC**: minimum inhibitory concentration. **FIC**: fractional inhibitory concentration.

Table 2. Interactions of vegetable extracts.

Interactions	Effect	
A. Caven and A. Furcatispina (A+B)	(0.75)	Additive
A. caven and P. torquata (A+ C)	(1.25)	Indifferent
A. Furcatispina and P. torquata (B+C)	(0.56)	Additive
A. caven and N. cataria (A+D)	(1.50)	Indifferent
A. furcatispina and N. cataria (B+D)	(2.50)	Indifferent
P. torquata and N. cataria (C+D)	(3.00)	Indifferent

(Number) denotes the ΣFIC index value.

Table 3. Detection biofilm-forming strains for quantitative microplate method.

Strain	Absorbance	Standard Deviation
L. grayi LG01	0.270	± 0.027
L. ivannovii	0.199	± 0.050
L. seeligeri LS01	0.222	± 0.057
L. monocytogenes LM01	0.340	± 0.017
L. monocytogenes 74910	0.131	±0.015
L. innocua 74915	0.219	± 0.043
L. innocua LI56	0.202	± 0.050
L. innocua LI02H	0.327	± 0.027
L. innocua LI1788	0.334	± 0.028
L. innocua LI1787	0.281	± 0.012
L. innocua LI1792	0.186	± 0.020
L. innocua LI1790	0.215	± 0.022
L. innocua LI1791	0.175	± 0.016
L. innocua LI1779	0.182	± 0.017
L. innocua LI1782	0.160	±0.014
**L. monocytogenes* CLIP 74904	0.375	± 0.039
**S. aureus* 11F2	0.442	± 0.074
**S. epidermidis* ATCC 12228	0.274	± 0.012
Control medium	0.056	± 0.010

Cutoff value OD550 ≥0.300 biofilm producer strain. **S. aureus* 11F2 (positive control), *L. monocytogenes* CLIP 74904 (positive control), *S. epidermidis* ATCC 12228 (negative control).

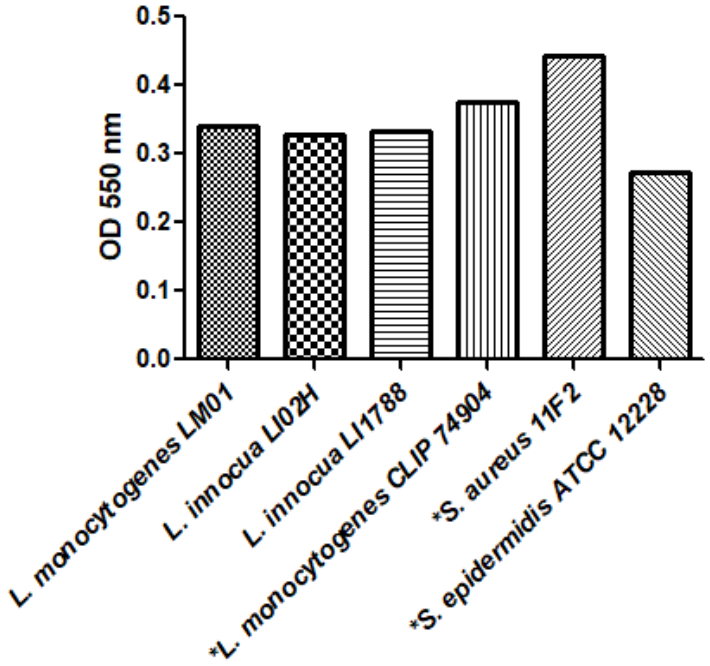

Figure 1. Biofilm producer strains (Cutoff value OD_{550} ≥0.300). *S. aureus* 11F2 (positive control), *L. monocytogenes* CLIP 74904 (positive control), *S. epidermidis* 12228 (negative control).