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Antimicrobial Activity from Cellular, Subcellular and Molecular Fractions of Cultured Microbes against Each Other

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Key words
Antimicrobial activity, Antagonism, Bacteria, Antibiotics, Ultrasonication.

ABSTRACT

Mankind is using antimicrobial agents in various forms since last 2000 years. The present study was designed to evaluate the antimicrobial activities of different culture microbes against each other. A total six bacteria including *Salmonella typhi, Escherichia coli, Pasteuralla multocida, Lactobacillus bulgaricus, Micrococcus luteus* and *Staphylococcus aureius* were used against each other to evaluate their antimicrobial activities. All of bacteria were cultured in LB media and further the culture of each bacterium was divided into three parts, the first part was centrifuged in order to collect supernatant. While the second and third parts were also centrifuged but however supernatants were discarded to get their pellets, which were dissolved in normal saline. Of this one part was taken for thaw and freeze treatment, while the second for ultrasonication treatment, before conducting antimicrobial activities. Zones of inhibition were measured by using disc diffusion method. Supernatant fraction of *E. coli* was positive against *Sal. typhi* and supernatant fraction of *P. multocida* was positive against *E. coli,* which show that antimicrobial agents were secretory in nature. Sonicated fractions were on second order to produce antimicrobial activities. The freeze thaw method was least effective in secreting active antimicrobial agents from tested bacteria. From the present study it is concluded that microbes are natural reservoir of antimicrobial compounds.

INTRODUCTION

Different species of streptomyces and actinomycetes are reported to produce many medicinally important compounds and they account for more than 70% of the naturally occurring antibiotics (Baskaran *et al*., 2016). The presently available drugs to combat infections caused by gram negative bacteria are not enough and there are still many antibiotics that are under clinical trials to fill the gaps and limitation in drug development. Initiative of infectious Disease Society of America is working on 10 novel antibiotics against bacilli, which will be helpful in combating infectious diseases (Taneja and Kaur, 2016).

Bacteria form biofilms in both natural and artificial like industrial environments. In such biofilms bacterial cells are linked together showing complex form of behaviors like coordination, communication, antagonism, cooperation and other communal behaviors and intercellular interactions (Moons *et al*., 2009). Bacteria form organized biofilms in the oral cavity on teeth and mucosal surfaces (Kolenbrander *et al*., 2010), which help in intercellular microbial interactions (Jakubovics *et al*., 2008). Antagonism in microbes has been worked out since previous century in the field of microbiology (Jay, 1996). Such interactions among microbes provide important and alternate method to treat infections (Stiles, 1996).

According to Cleveland *et al*. (2001) bacteria produce a number of antimicrobial proteins as a natural defense to kill or inhibit other bacteria. However nature, functions, structures and mode of action of such bacteriocin need to be explored. Lazdunski (1988) was the first who reported a bacteriocin in gram negative bacteria. Then other

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antimicrobial were reoprted like Enterocin 4 (Nunez *et al*., 1997), Linocin M-18 (Eppert *et al*., 1997), Pediocin AcH (Baccus-Taylor *et al*., 1993), Enterocin (Aymerich *et al*., 2000) etc. These antimicrobial peptide and protein have many applications but bacteria by their self-produced these to kill other bacteria.

Antibiotics and antimicrobial discovery is a difficult job even today when many of antibiotics has been discovered. According to Scientists still we need to discover hundreds of antimicrobial agents. This study was designed to screen for natural antibiotics and new templates for synthetic antibiotics from bacteria. Furthermore it aimed to evaluate antimicrobial activity of different cultured bacteria against each other.

MATERIALS AND METHODS

The cultured bacteria were collected from Biotechnology Laboratory, PMAS Arid Agriculture University Rawalpindi Pakistan and were refreshed on LB media. A total six bacteria *i.e. E. coli, Sal. typhi, P. multocida, S. aureius, M. luteus* and *L. bulgaricus* were used in this study. All these bacteria were further sub-cultured on LB media and density of cultures were adjusted at 0.4 by taking $O.D_{600}$. Further the cultures were divided into three parts, the first part was centrifuged at 10,000 rpm at 4 °C for 10 min and its supernatant was taken. The second and third parts was also subjected to centrifugation at 10,000 rpm at 4 °C for 10 min but their supernatants were discarded and pellets were collected in sterile PBS (pH 7.4). The collected pallet samples were further divided into two parts, the first part was subjected to ultrasonication treatment and second part was subjected to freeze and thaw treatment.

Antimicrobial assay

For this purpose sterilized whatman filter paper discs were soaked in the treated samples of each bacterium, like some in harvested supernatant samples of bacteria, some in thaw and freeze samples, while other ultrasonicated samples in. The rest of the bacteria were now streaked on agar plates after maintaining density at $O.D_{600}$ at 0.4 and incubated at 37 °C for 1 h. After this incubation period, the presoaked discs were transferred onto streaked agar plates and then incubated for 16 h at 37°C. The presoaked discs from chloramphenicol were used as positive control. After incubation the results were visualized, carefully and their photographs were captured.

Minimum inhibition concentrations (MIC)

For this purpose activities conferring samples were diluted till the last effective concentrations against bacteria. INT was used as control in calculating MIC from different antimicrobial conferring samples.

Statistical analysis

Polynomial ANNOVA was used for analysis of variance with keeping significancy level at 95%.

RESULTS AND DISCUSSION

Bacteria possess ability to ensure their survival inside the host by producing various compounds like toxins and effectors etc. These secretions enable them in colonization over a substratum (Kim *et al*., 2010). When the bacteria colonize a substratum and form biofilms, they exhibit various sort of interactions, like antagonism and coordination by producing various compounds (Moons *et al*., 2009). According to Field *et al*. (2016) the spreading of more and more pathogenic bacteria are becoming resistant to the existing antibiotics. To compensate this antimicrobial resistance there is need of novel drugs, antibiotics and other strategies to control various diseases. They reported the antimicrobial activity of Nisin (an antimicrobial peptide belonging to the family of the lantibiotic) against Gram-positive bacteria. Keeping in view these aspects of intercellular interactions among bacteria, the present study was designed to evaluate antimicrobial activities of bacteria against each other.

In some studies the bacterial interactions was studied by using through stoichiometric constraint based modeling (Stolyar, 2007). To study antimicrobial interaction among six common microbes, three methods were applied to study this interaction. The protocol is designed in such a way that the level of interaction should get highlight, the protocol help in elucidating the nature of antimicrobial agent.

A few studies reported on the antimicrobial activities of bacterial against each other. However enough work has been done to understand the intercellular microbial interactions. The work of Patricia *et al*. (2014) favors our studies. They worked on the synergistic relationship between *Candida albicans* and oral streptococci, which are an example of bacterial-fungal interaction. The understanding of such interactions will be helpful in treatment of many diseases and will improve human health.

Antimicrobial activity of E. coli

During the present study supernatant fraction of *E. coli* showed higher activity against *P. multocida* (0.5cm), which means their antimicrobial agents are secretory in nature. Antimicrobial activity against *S. typhi* was recorded in its sonicated (0.25) followed by supernatant (0.25)

product while no activity recorded in thaw freeze product. This shows that antimicrobial agent could be secretory as well as membrane bounded but not intercellular. Similarly *E. coli* also showed antimicrobial activity against *S. aureus* in its sonicated product (0.1cm) and supernatant (0.2cm), against *L. bulgaricus* in its sonicated product (0.1cm) and supernatant (0.2cm), against *M. lutius* in its sonicated product (0.1cm), in supernatant (>0.1cm) and (1.3cm) against control (Table I). Flynn *et al*. (2002) reported the first bacteriocin form a human probiotic bacterium *i.e. Lactobacillus salivarius* subsp. *salivarius* UCC118. This novel bacteriocin showed good antimicrobial activities. They also studied its genetic basis, and reported genes that encode or enhance its production. In our reported studies as antimicrobial bacterions were not identified as this was not as a part of our studies. Further work is needed to investigate nature these bacteriocins as well as to find their genetic basis.

Table I.- Antimicrobial activity from different fractions of *E. coli.*

Organism	Supernatant	Sonicated	Freeze and thawed	Positive control
S. typhi	0.25	0.25	0.00	1.25
S. aureus	0.22	0.12	0.00	1.25
P. multocida	0.51	0.00	0.00	1.15
L. bulgaricus	0.22	0.13	0.00	0.95
M. lutius	0.15	0.12	0.00	1.10

Mean values in columns and rows are statistically non-significant (P>0.05) in comparison to the control group.

Table II.- Antimicrobial activity from different fractions of *S. typhi.*

Organism	Supernatant	Sonicated	Freeze and thawed	Positive control
E. coli	0.22	0.71	0.00	1.30
S. aureus	0.11	0.12	0.00	1.25
P. multocida	0.00	0.11	0.00	1.00
L. bulgaricus	0.11	0.35	0.05	1.00
M. lutius	0.12	0.60	0.00	1.10

Mean values in columns and rows are statistically non-significant (P>0.05) in comparison to the control group.

Antimicrobial activity of S. typhi

Higher antimicrobial activity against *E. coli* recorded in its sonicated product (0.7cm) followed by its supernatant (0.2cm). It also showed activity against *S. aureus* (>0.1cm) both in sonicated and supernatant, against *P. multocida* in sonicated (0.1cm), against *L. bulgaricus* in supernatant (>0.1), in sonicated (0.1), in Freeze and thaw (0.35cm), against *M. lutius* in sonicated (0.6cm), in supernatant (>0.1cm) and (1.35cm) against control (Table II).

Antimicrobial activity of S. aureus

A higher activity against *E. coli* recorded in sonicated (0.65cm), supernatant (0.525cm), while in Freeze and thaw (0.325cm). It also has activity against *S. typhi* in sonicated (0.5cm), supernatant (0.32cm), against *P. multocida* in sonicated (0.325cm), supernatant (0.425cm), F/T (0.2cm), against *L. bulgaricus* in sonicated (0.3cm), supernatant (0.2cm), F/T (0.4cm), against *M. lutius* in sonicated (0.5cm), supernatant (0.25), F/T (0.25cm) and against control (1cm) (Table III).

Table III.- Antimicrobial activity from different fractions of *S. aureus.*

Organism	Supernatant	Sonicated	Freez and thawed	Positive control
E. coli	0.53	0.65	0.32	1.30
S. typhi	0.32	0.51	0.00	1.25
P. multocida	0.43	0.33	0.21	1.00
L. bulgaricus	0.20	0.30	0.43	1.00
M. lutius	0.25	0.51	0.25	1.10

Mean values in columns and rows are statistically non-significant (P>0.05) in comparison to the control group.

Antimicrobial activity of P. multocida

It showed maximum activity against *E. coli* (0.975cm) in its supernatant, in sonicated (0.575cm), while in freeze and thaw (0.85cm). It also showed activity against *S. typhi* is in supernatant (0.5cm), in sonicated (0.1cm), against *S. aureus* in supernatant (0.375), against *L. bulgaricus* in F/T (0.275cm), against *M. lutius* in supernatant (0.225cm), in F/T (0.1cm) and (1cm) against control (Table IV).

Table IV.- Antimicrobial activity from different fractions of *P. multocida.*

Organism	Supernatant	Sonicated	Freeze and thawed	Positive control
E. coli	0.98	0.57	0.85	1.35
S. typhi	0.50	0.11	0.00	1.22
S. aureus	0.38	0.00	0.00	1.31
L. bulgaricus	0.00	0.00	0.27	1.00
M. lutius	0.22	0.00	0.10	1.00

Mean values in columns and rows are statistically non-significant (P>0.05) in comparison to the control group.

Antimicrobial activity of L. bulgaricus

It exhibited activity only against *S. aureus* (0.15cm) in its sonicated product, against *P. multocida* in supernatant (0.1cm) and against control (1.25cm) (Fig .1, Table V).

Zahid *et al*. (2015) used five lactic acid bacteria *i.e. L. acidophilus, L. bulgaricus, L. dulbrueckii, L. plantarum* and *L. fermentum* to isolate bacteriocin from them by using ammonium sulphate precipitation method. By testing these bacteriocin against Methicillin-Resistant *Staphylococcus aureus*, *E.coli, Salmonella* and *Staphylococcus aureus,* these given excellent antimicrobial activities against these pathogen. They argue that such antimicobial bacteriocin can be used to treat a variety of huffmann diseases. Their reported works favor the present studies and also highlight the importance of the reported work.

Table V.- Antimicrobial activity from different fractions of *L. bulgaricus.*

Organism	Supernatant	Sonicated	Freeze and thawed	Positive control
E. coli	0.00	0.00	0.00	1.37
S. typhi	0.00	0.00	0.00	1.22
S. aureus	0.00	0.15	0.00	1.20
P. multocida	0.10	0.00	0.00	1.21
M. lutius	0.00	0.00	0.00	1.00

Mean values in columns and rows are statistically non-significant (P>0.05) in comparison to the control group.

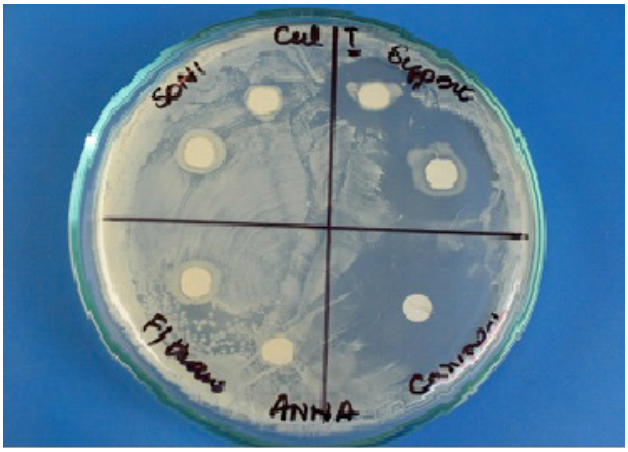

Fig. 1. Activity of *L. bulgaricus* against *S. aureus* in sonicated product.

Antimicrobial activity of M. leutius

It showed a higher activity against *E. coli* in its freeze and thaw (0.7cm), in supernatant (0.1cm), against *S. typhi* in F/T (0.35cm), against *S. aureus* in F/T (0.55cm), against *L. bulgaricus* in supernatant (>0.1) and (1.3cm) against control (Table VI). The work of Nielsen *et al*. (1990) also favors the present findings, who reported the same work but with different microbes from that in our studies. They used a bacteriocin produced by *Pediococcus acidilactici* which showed an inhibitory and bactericidal activity against *Listeria monocytogenes*. According to Li and Gu (2016), *Lactobacillus plantarum ZJ95* is an important probiotic of infants, which is reported to produce riboflavin, that has antimicrobial activity. They sequenced its genome in order to facilitate the biosynthesis of this bacteriocin and to highlight its importance. The recorded antimicrobial activity of different microbes, in the present study may also be proven helpful in treatment of various human diseases and other industries also. Guinane *et al*. (2016) studied that gastrointestinal microbiota produce a variety of active substances like bacteriocins, which have antimicrobial activity, which enhance human health. They isolated a novel broad spectrum class IId bacteriocin by gut *Lactobacillus salivarius,* which has regulating effect on the gut microbiota like Bacteroides, Clostridium and *Bifidibacterium* spp. According to Joerger (2003), Bacteria produce the bacteriocins (proteinaceous in nature) which are lethal against those nonproducing bacteria. This property of normal micro-flora in the gut of animals is useful the competitive exclusion of pathogenic bacteria. Similarly the purified or partially purified bacteriocins could be used as preservatives agents. These suggestions highlight the importance of the present work, in which *in vitro* activities were performed on various strains of the bacteria to test their antagonistic actions against each other. Further work is needed to isolate the bacteriocins and their nature, mode of action and potency.

Table VI.- Antimicrobial activity from different fractions of *M. lutius.*

Organism	Supernatant	Sonicated	Freeze and thawed	Positive control
E. coli	0.11	0.00	0.71	1.37
S. typhi	0.00	0.00	0.35	1.20
S. aureus	0.00	0.00	0.55	1.20
P. multocida	0.10	0.00	0.00	1.20
L. bulgaricus	0.12	0.00	0.00	1.00

Mean values in columns and rows are statistically non-significant (P>0.05) in comparison to the control group.

CONCLUSIONS

This study proved that bacteria are good source of natural antimicrobial agent(s). The use of bacteria in

medicine and biotechnology industry is already common. Huge quantities of antibiotics and other antimicrobial agents are produced from naturally occurring bacteria as well as from genetically modified bacteria. Different microbes have different modes of action against other microbes, some act as microbicidal, some act as microbistatic. Similarly, some antimicrobial agents are organic acid in nature, some are oil/lipid in nature, some are proteins like bactericin, some are bioactive peptides, some produced inorganic acids or toxic gases to control microbes etc. The microbes selected in current study are common pathogens of class mammalia and aves. All of these microbes are well reported in economic losses of livestock and poultry industry. The antimicrobial resistance against currently used antibiotics further highlights the importance of this study. There is need of natural, synthetic and semi synthetic antimicrobial drugs in market. Bacteria in considered as cheap and effective source of new antimicrobial agents and clear zones of inhibitions produced by *E.coli, S. aureus* and *P. multocida,* against each other and other microbes provide a big support to this idea. Microbes are responsible to cause diseases in livestock and poultry industry and on the same time they can provide remedy against other microbes through their antimicrobial agent(s).The prolonged use of antimicrobial agents can affect gut microbial flora. The use of probiotics is highly recommended after prolonged used of antimicrobial to replenish the lost gut flora.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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