

## Antimicrobial activities of *Pseudomonas* spp. strains isolated from raw milk collected in Turkey

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### Abstract:

In the study, a total of fifteen *Pseudomonas* spp. strains were analysed. All the strains were isolated from raw milk samples collected from Kayseri and Nigde provinces in Turkey. *Pseudomonas* spp. were characterized to species level with the use of analytical profile index. The antimicrobial activity studies were investigated by using agar-well diffusion method. From the results, it was determined that *P. aeruginosa* and *E. coli* were significantly inhibited by *Pseudomonas* strains. Also, it was found that *Pseudomonas* strains had shown inhibition effect on lactic acid bacteria and lactic acid bacteria had significantly high inhibition effect on *Pseudomonas* strains.

**Key Words:** *Pseudomonas*, isolation, raw milk, antimicrobial activity, Lactic Acid Bacteria

### Introduction

Milk is highly susceptible to contamination and can serve as an efficient means of infecting human pathogens, especially gram-negative bacteria, because they are widely distributed in the environment (Garedew et al., 2012). Microbial contamination of milk begins by milking. The most important sources of contamination are animal breast, skin, hair, human hand, milking machines, milk tanks and coolers. Microorganisms infected through air, dust, soil, water and fertilizer from these environments to milk (Akoğlu, 2006). In other words, high counts of psychrotrophic bacteria in raw milk are directly related to poor hygienic conditions during production and milking, and to the time and temperature of milk storage (de Almeida et al., 2017).

*Pseudomonas* has been identified as predominant milk-associated psychrotrophic bacteria, making it one of the most important bacterial groups in the dairy industry (Wiedmann et al., 2000; Marchand et al., 2009). The most commonly detected *Pseudomonas* species in milk and milk products are *P. fluorescens*, *P. gessardii*, *P. fragi*, and *P. lundensis* (Mallet et al., 2012). *Pseudomonas* spp. can grow over a temperature range of 4–42°C, with an optimal growth temperature above 20°C (Chakravarty and Gregory, 2015). They are present in different environments and are frequently linked to food spoilage, especially, that of raw milk (Quigley et al., 2013; Chakravarty and Gregory, 2015).

Bacterial resistance to antibiotics poses a serious challenge to the prospect of chemotherapy, because the traditional antibiotics and its derivatives are becoming nonfunctional. The whole world is thus confronted with a looming drug crisis which has motivated the pursuit of new antibiotic compounds with

novel mechanisms of action (Sengupta, 2012). The bacterial products have served the development of new pharmaceutical drugs that are widely used to fight bacterial infections (Bredholdt et al., 2007). Antagonism between the bacteria is well-known phenomenon Gratia's essay published in 1925 (Gratia, 1925). Antimicrobial drug-producing micro-organisms are dominant over other species through agencies (Padilla et al., 2001). The species of the genus *Pseudomonas* rhizosphere antagonistic activities were quite good. Bacteriocin-like antibiotics produced by bacteria from genus *Pseudomonas* and fenazin (Gram, 1993), hydrogen cyanide (Castric, 1977), antibiotics and sideroforlar (Fakhouri et al., 2001), such as antimicrobial agents were involved in the suppression of many root pathogens. Also some researchers explained that *Pseudomonas* spp. strains have showed antimicrobial effect against some pathogen and contaminant bacteria (Dopazo et al., 1988, Gram, 1993, Gram and Melchiorsen, 1997) .

The aims of the present work were (i) to isolate and identify (analytical profile index (API 20 NE)) *Pseudomonas* spp. from the raw milk samples; (ii) to investigate of the inhibition activity of *Pseudomonas* spp. strains on some pathogen test bacteria and some lactic acid bacteria; and (iii) also to determine of inhibition effect of lactic acid bacteria on *Pseudomonas* spp. strains.

## Materials And Methods

### Sample collections

A total of 50 samples of raw milk collected from Kayseri and Nigde provinces in Turkey. The samples maintained at low temperature during transfer to the laboratory and analyzed within 24 hrs. Each sample was collected in sterial bags to minimize the possibility of contamination and maintained at low temperature during transfer to the laboratory and analyzed within 24 hrs.

### Media and test bacteria

In studies of bacteria isolation, *Pseudomonas* Selective CN, CFC Agar Base (Merck 1.07620), McConkey Agar (Merck) and Cetrimide Agar Medium (Merck, Darmstadt, Germany) were used. In studies of antimicrobial activity, MRS Broth (Oxoid), MRS Agar, Nutrient Broth (Sigma-Aldrich) and Nutrient Agar Media were used.

In the studies, *Bacillus subtilis* RSKK 244, *Bacillus subtilis* 1404, *Bacillus subtilis*, 2362, *Salmonella* 21.3, *Pseudomonas aeruginosa* ATCC 27853, *Shigella sonnei* RSKK 877, *Staphylococcus epidermidis*, *Bacillus thuringiensis*, *Bacillus megaterium* RSKK 5117, *Bacillus cereus* 863, *Staphylococcus aureus* Koag (+), *Escherichia coli* ATCC 35218 were used as test bacteria, *Lactobacillus brevis* Z.20L, *Lactobacillus helveticus* 75.L., *Lactobacillus fermentum* DSMS 23271, *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus plantarum* ATCC 20246, *Lactobacillus acidophilus* ATCC 53103 were used as test bacteria.

### Isolation of Bacteria

50 raw milk samples were diluted with sterile physiological water. Homogeneous samples were diluted serially from  $10^{-1}$  to  $10^{-7}$  and 0.1 ml samples were inoculated on McConkey agar plates from dilutions of  $10^{-3}$  and incubated at 37 ° C for 24 hours. Lactose (–) colonies on McConkey Agar were picked up and plated on *Pseudomonas* CN, *Pseudomonas* CFC and Cetrimit agar at 37 ° C for 24 –48 hrs. After incubation random choices made by Gram staining and examined under a microscope

The isolates were sub-cultured on the same medium until pure cultures were achieved. The isolated bacteria were grown in Nutrient Broth and were then stored in 30.0% (v/v) glycerol at–80 ° C and used as stock cultures in subsequent analysis.

### Identification of Bacteria

The isolated bacteria were evaluated firstly the colony structure, the gram-stain, catalase activity, +4<sup>0</sup> and + 42<sup>0</sup>growing. *Pseudomonas* spp for a further description was defined as strains with the use of

analytical profile index (API Count NE 20 /; Biomerieux, Marcy l'Étoile, France) were characterized to species level. 15 % glycerol identified strains have been preserved in -20 ° C. Stocks renewed every two months.

### ***Pseudomonas* spp. strains inhibitory effect on the contaminant and pathogenic microorganisms**

The determination of the inhibitory effect of *Pseudomonas* spp. strains isolates on test bacteria was carried out according to the agar-well diffusion method (Reinheimer et al.,1990). All bacteria were cultured in Nutrient broth medium and incubated at the appropriate temperature for 24 h. Nutrient agar medium (20 ml) was poured into each sterile Petri dish (100mm diameter). Suspensions (100 ml) of target strain cultured for 24 h were spread on the plates, and wells of 6mm diameter were punched in the agar with a sterile steel borer. The *Pseudomonas* strains were centrifuged at 6000g for 15 min to remove cell debris. At the end of the centrifuge (supernatant) with a 0,45 µm disposable filter was sterilized by microfiltration. After, supernatant samples (100 ml) were filled into the wells of agar plates directly. Each sample (100 ml) was then filled into the wells of agar plates inoculated with test bacteria. The inoculated plates were incubated for 24 h at their optimum growth temperatures, and the diameter of the inhibition zone was measured with calipers as mm. The measurements were done basically from the edge at the zone to the edge of the wall.

### ***Pseudomonas* spp. strains inhibitory effect on the some lactic acid bacteria**

Lactic acid bacteria were cultured in MRS medium and appropriate incubation temperature. The determination of the inhibitory effect of *Pseudomonas* spp. strains on lactic acid bacteria was carried out according to the agar-well diffusion method.

### **Some lactic acid bacteria inhibitory effect on *Pseudomonas* spp. strains**

Bacteria belonging to the genus *Pseudomonas* were used as the test bacteria. The test bacteria were cultured in Nutrient broth medium and appropriate incubation temperature. Inhibition effect was determined by agar-well diffusion method.

## **Results And Discussion**

A total of 15 *Pseudomonas* isolates was obtained from raw milk. These strains identified by using API 20 NE (Table 1). API 20 NE provided good identification of dairy *Pseudomonas* isolates to the species level (Wiedmann et al., 2000).

**Table 1.** Isolated species and their geographical origin

<b>Number</b>	<b>Strain</b>	<b>Species</b>	<b>Origin</b>
<b>1</b>	H <sub>1</sub>	<i>P. luteola</i>	Samples of raw milk in Kayseri Province
<b>2</b>	H <sub>2</sub>	<i>P. paucimobilis</i>	Samples of raw milk in Kayseri Province
<b>3</b>	H <sub>3</sub>	<i>P. vesicularis</i>	Samples of raw milk in Niğde Province
<b>4</b>	H <sub>4</sub>	<i>P. vesicularis</i>	Samples of raw milk in Niğde Province
<b>5</b>	H <sub>5</sub>	<i>P. vesicularis</i>	Samples of raw milk in Niğde Province
<b>6</b>	H <sub>6</sub>	<i>P. vesicularis</i>	Samples of raw milk in Kayseri Province

7	H <sub>7</sub>	<i>P. vesicularis</i>	Samples of raw milk in Niğde Province
8	H <sub>8</sub>	<i>P. vesicularis</i>	Samples of raw milk in Niğde Province
9	H <sub>9</sub>	<i>P. aeruginosa</i>	Samples of raw milk in Niğde Province
10	H <sub>10</sub>	<i>P. fluorescens ssp. indolegenes</i>	Samples of raw milk in Niğde Province
11	H <sub>11</sub>	<i>P. fluorescens ssp. indolegenes</i>	Samples of raw milk in Niğde Province
12	H <sub>12</sub>	<i>P. fluorescens ssp. indolegenes</i>	Samples of raw milk in Niğde Province
13	H <sub>13</sub>	<i>P. fluorescens ssp. indolegenes</i>	Samples of raw milk in Niğde Province
14	H <sub>14</sub>	<i>P. fluorescens ssp. indolegenes</i>	Samples of raw milk in Niğde Province
15	H <sub>15</sub>	<i>P. fluorescens ssp. indolegenes</i>	Samples of raw milk in Niğde Province

This study examined the antimicrobial activity of 15 *Pseudomonas* spp. strains against tested bacteria. Our isolates showed antimicrobial activity against both Gram (+) and Gram (-) bacteria. These inhibition activity results are given in Table 2.

In our study it is found that 47% of all *Pseudomonas* spp. strains showed antimicrobial activity on *Salmonella* test bacteria. Also, *P. aeruginosa* H<sub>9</sub> strain has beter antimicrobial effect of 18.2 mm diameters against *Salmonella*.

The other significant findings of our study was 87 % *Pseudomonas* spp. strains had inhibition effect on *P. aeruginosa* strains. All the *Pseudomonas* strains except *P. luteola* H<sub>1</sub> and *P. vesicularis* H<sub>5</sub> showed inhibitoric effect on *P. aeuginosa* ATCC 27853. Some research revealed that antimicrobial substances belonging to *Pseudomonas* genus had inhibitoric effect on other *Pseudomonas* strains (Jones et al., 1974; Fermor and Lynch, 1988; Lavermicocca et al., 1999).

The results indicated that 20% of these strains had antimicrobial activity on *B. subtilis* 2362, *B. subtilis* RSKK 244 and *B. megaterium* strains while 13% of *Pseudomonas* spp. had inhibitoric effect on *B. thuringiensis*. However only 7% of our strains showed inhibition effect on *B. cereus* 863 and *B. subtilis* 1404.

Some researchers reported 5 % of total 19 *P. aeruginosa* strains showed antimicrobial effect on *B. subtilis* strains (Jeppesen, 1995). Also, Vachée and colleagues revealed that 16.6% of 54 *Pseudomonas* spp. strains had inhibition on *B.cereus* (Vachée et al., 1997).

In the study 20% of *Pseudomonas* spp. showed inhibitory effect on *Shigella* and *Staphylococcus epidermidis* strains. Also, 19% of *Pseudomonas* spp. had antimicrobial effect against *S. aureus* Koag (+) test bacteria. Oblinger and Kreft determined that *Pseudomonas* spp. strains had antimicrobial activity on *S. aureus* (Oblinger and Kreft, 1990).

In our study 33% of *Pseudomonas* spp. strains had antimicrobial activity on *E. coli* ATCC 35218. Padilla et al., explained that 12% of *P. aeruginosa* strains showed inhibitory effect on *E. coli* (Padilla et al., 2001). Several researchers indicated that only 15 number of total 209 *Pseudomonas* spp. strains were

isolated from fish showed antimicrobial effect on *E. coli* strains (Gram, 1993). These informations is similar to the results of our study.

**Table 2.** Inhibition of *Pseudomonas* strains showing antimicrobial activity on some pathogens and contaminant bacteria (inhibition zone diameter mm)

<b>*Inhibition zone (diameter, mm) against tested bacteria</b>												
<i>Pseudomonas</i> strains	<i>Salmonella</i>	<i>P.aeruginosa</i> ATCC 27853	<i>B. subtilis</i> 2362	<i>B. subtilis</i> 1404	<i>Shigella</i>	<i>S. epidermidis</i>	<i>B. thurugiensis</i>	<i>B. megaterium</i>	<i>B. cereus</i> 863	<i>B. subtilis</i> RSKK 244	<i>Staph. aureus</i> Koag (+)	<i>E. coli</i> ATCC 35218
<i>P. luteola</i> H <sub>1</sub>	10.8 ±2.6	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
<i>P. paucimobilis</i> H <sub>2</sub>	11.3±2.3	8.7±1.5	10.4±4.8	NI	8.2±0.6	8.2±0.6	7.7±1.1	4.9±1.7	NI	6.3±1.7	4.8±0	7.5±3.9
<i>P. vesicularis</i> H <sub>3</sub>	NI	7.4±7.4	NI	NI	NI	NI	NI	NI	NI	NI	NI	2.3±2.3
<i>P. vesicularis</i> H <sub>4</sub>	9.3±1.1	5.6±5.6	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
<i>P. vesicularis</i> H <sub>5</sub>	NI	NI	3.1±3.1	NI	NI	NI	NI	NI	NI	NI	NI	NI
<i>P. vesicularis</i> H <sub>6</sub>	3.4±3.4	7.1±7.1	NI	NI	NI	3.8±3.8	NI	6.7±2.5	NI	3.9±3.9	NI	1.4±1.4
<i>P. vesicularis</i> H <sub>7</sub>	NI	9.9±1.3	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
<i>P. vesicularis</i> H <sub>8</sub>	5.6±0.8	4.3±4.3	NI	NI	4.7±1.9	NI	NI	NI	NI	NI	NI	NI
<i>P. aeruginosa</i> H <sub>9</sub>	18.2±2	8.0±1.4	12.7±3.8	11.3±0.9	10.4±2.2	10.4±2.2	9.1±1.5	9.3±0.5	7.1±1.3	7.7±1.1	6.4±0.4	10.1±1.3
<i>P. fluorescens</i> ssp. <i>indolegens</i> H <sub>10</sub>	NI	1.8±1.8	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
<i>P. fluorescens</i> ssp. <i>indolegens</i> H <sub>11</sub>	NI	21.1±2.7	NI	NI	NI	NI	NI	NI	NI	NI	NI	7.7±7.7
<i>P. fluorescens</i> ssp. <i>indolegens</i> H <sub>12</sub>	NI	6.9±6.9	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
<i>P. fluorescens</i> ssp. <i>indolegens</i> H <sub>13</sub>	NI	13.1±0.9	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
<i>P. fluorescens</i> ssp. <i>indolegens</i>	NI	17.0±6.4	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

<i>s H<sub>14</sub></i>												
<i>P. fluorescens ssp. indolegenes H<sub>15</sub></i>	8.8±8.8	15.3±4.3	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

*Pseudomonas* bacteria is important contaminant microorganisms for food products such as milk, chicken, meat and fish (Kıvanç, M., 1990; Flint and Hartley, 1996). The many studies of relationship between other microorganisms and *Pseudomonas* spp in foods are very limited. Also there have been contrasting opinions with each other.

Our study revealed that antimicrobial effects (3.4-12.6 mm) on lactic acid bacteria of *Pseudomonas* strains (Table 3). Freedman et al. found that *Pseudomonas* strains isolated from plants and foods stimulated siderophore production in King's B medium agar and these strains showed inhibition effect on lactic acid bacteria by increasing the inhibitor activity (Freedman et al., 1989). However many other studies have been reported that they can increase the growth of lactic acid bacteria although *Pseudomonas* strains have inhibitory effect on many microorganisms (Gram, 1993, Gram et al, 2002).

**Table 3.** Antimicrobial effect on Lactic Acid Bacteria of some *Pseudomonas* spp. strains

<b>*Inhibition zone (diameter, mm) against Lactic Acid Bacteria</b>					
<i>Pseudomonas</i> strains	<i>L. fermentum</i> DSMS 23271	<i>L. acidophilus</i> ATCC 4356	<i>L. plantarum</i> ATCC 20246	<i>L. helveticus</i> 75. L	<i>L. Brevis</i> Z. 20L.
<i>P. luteola</i> H <sub>1</sub>	NI	6.3±6.3	NI	NI	NI
<i>P. vesicularis</i> H <sub>4</sub>	6.1±6.1	NI	NI	NI	NI
<i>P. vesicularis</i> H <sub>5</sub>	NI	NI	NI	NI	3.4±3.4
<i>P. aeruginosa</i> H <sub>9</sub>	8.3±0.1	NI	NI	NI	NI
<i>P. fluorescens ssp. indolegenes</i> H <sub>10</sub>	5.6±0.2	NI	NI	NI	NI
<i>P. fluorescens ssp. indolegenes</i> H <sub>11</sub>	7.9±0.7	NI	12.6±0.8	8.0±8.0	7.3±1.3
<i>P. fluorescens ssp. indolegenes</i> H <sub>12</sub>	8.6±2.2	9.0±1.8	12.3±0.3	10.4±10.4	11.4±0.4
<i>P. fluorescens ssp. indolegenes</i> H <sub>13</sub>	NI	NI	NI	NI	8.6±2.4
<i>P. fluorescens ssp. indolegenes</i> H <sub>14</sub>	8.7±1.5	NI	NI	NI	7.4±2.0
<i>P. fluorescens ssp. indolegenes</i> H <sub>15</sub>	NI	NI	NI	NI	NI

NI: No Inhibition.

\*Values are the means ± standard deviations of triplicate measurements.

We also studied antimicrobial effects on *Pseudomonas* strains of lactic acid bacteria. Lactic acid bacteria is industrial importance microorganisms because of health and nutritional benefits and fermentative ability (Simsek and Bilgin, 1996). Lactic acid bacteria are gram-positive bacteria defined product of lactic



acid and characterized of glucose to lactic acid to translate. Lactic acid bacteria is important and economically valuable for food preparation, food processing and creation of food derivatives. These microorganisms have lots of ability such as lactic acid production, protein hydrolysis and synthesis of aromatic compounds (Baltasar et al, 1990). Researchers have reported that lactic acid bacteria prevents lots of development of contaminant and pathogen microorganisms including *Pseudomonas* genus of bacteria in being various food products and it is responsible for lactic acid, hydrogen peroxide, diacetyl and low molecular weight and heat- resistant bacteriocin (Reinheimer et al., 1990; Aroutcheva et al., 2001). These antimicrobial agents play an important role in securing food products has been reported (Daeschel 1989; Zhu et al.,2000). In our study observed that all the six species of lactic acid bacteria examined showed antimicrobial activity (3.0-18.3 mm) against *Pseudomonas* strains (Table 4). We determined that among the lactic acid culture *L. helveticus* and *L. brevis* had the greatest antimicrobial activity against all the *Pseudomonas* strains.

**Table 4.** Antimicrobial effect on some *Pseudomonas* spp. strains of Lactic Acid Bacteria

*Inhibition zone (diameter, mm) against <i>Pseudomonas</i> spp.															
Lactic Acid Bacteria	<i>P. luteola</i> H <sub>1</sub>	<i>P. paucimobis</i> H <sub>2</sub>	<i>P. vesicularis</i> H <sub>3</sub>	<i>P. vesicularis</i> H <sub>4</sub>	<i>P. vesicularis</i> H <sub>5</sub>	<i>P. vesicularis</i> H <sub>6</sub>	<i>P. vesicularis</i> H <sub>7</sub>	<i>P. vesicularis</i> H <sub>8</sub>	<i>P. aeruginosa</i> H <sub>9</sub>	<i>P. fluorescens</i> ssp. <i>indologenes</i> H <sub>10</sub>	<i>P. fluorescens</i> ssp. <i>indologenes</i> H <sub>11</sub>	<i>P. fluorescens</i> ssp. <i>indologenes</i> H <sub>12</sub>	<i>P. fluorescens</i> ssp. <i>indologenes</i> H <sub>13</sub>	<i>P. fluorescens</i> ssp. <i>indologenes</i> H <sub>14</sub>	<i>P. fluorescens</i> ssp. <i>indologenes</i> H <sub>15</sub>
<i>L. fermentum</i> DSMS 23271	NI	NI	7.1±0.9	NI	NI	NI	NI	NI	6.0±1.2	7.6±4.2	NI	5.8±0.8	NI	3.6±3.6	NI
<i>L. acidophilus</i> ATCC 4356	NI	NI	9.5±2.5	NI	NI	NI	NI	NI	5.1±0.5	NI	NI	10.7±1.5	NI	12.9±4.1	NI
<i>L. plantarum</i> ATCC 20246	NI	NI	NI	NI	6.0±0.6	6.9±0.3	NI	6.8±1.4	10.4±1.8	NI	NI	6.2±0.8	NI	NI	NI
<i>L. helveticus</i> 75. L	9.3±2.5	13.0±0.2	11.0±2.2	15.7±0.3	18.3±2.3	8.1±0.7	8.1±1.5	8.3±0.9	9.5±0.3	5.5±1.1	10.2±2.0	11.1±2.3	7.9±1.7	5.1±5.1	5.9±0.5
<i>L. brevis</i> Z. 20L.	3±3	10.8±2.6	7.8±1.4	8.8±1.4	14.0±0.2	7.1±0.9	5.6±1.0	6.1±1.5	7.7±2.2	4.5±0.3	11.5±1.9	11.2±2.4	12.0±0.8	11.5±2.1	8.7±1.3
<i>L. acidophilus</i> ATCC 53103	NI	NI	8.5±3.5	NI	10.8±0	12.2±1.2	NI	11.1±0.7	5.7±0.5	8.0±0.4	9.2±2.0	11.5±0.5	NI	NI	NI

NI: no inhibition.

\*Values are the means ± standard deviations of triplicate measurement.

The lactic acid bacteria appears to have great inhibition effect on *Pseudomonas* spp. as food contaminant bacteria known provides significant benefits in the food industry. Also, microbial natural products still appear as the most promising source of the future antibiotics that society is expecting. The process of antibiotic discovery from natural products is complex and difficult. Therefore the benefits of our isolates and the chemical characterization of the antimicrobials determined are subject to further studies.

Nowadays investigation of antimicrobial activity with broad types of microorganisms and the discovery of new and more effective antibiotics as an alternative to antibiotics used in the treatment of different diseases has gained importance. According to the results of our study, *Pseudomonas* spp. strains showed antimicrobial activity especially on Gram negative test bacteria. In addition, it is very important for lactic acid bacteria to have an inhibitory effect on food contaminants, *Pseudomonas* strains, in terms of food safety.

Microbial natural products still appear as the most promising source of the future antibiotics that society is expecting. The process of antibiotic discovery from natural products is complex and difficult. Therefore the benefits of our isolates and the chemical characterization of the antimicrobials determined are subject to further studies.

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