Determination of Relationship Between *Satureja hortensis* L. Essential Oil Susceptibility of *Bacillus cereus* Strains and Their Fatty Acid Methyl Ester Profiles

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NESLIHAN DIKBAS*\(^{a}\), KENAN KARAGÖZ\(^{b}\), FATIH DADAŞOĞLU\(^{c}\), RECEP KOTAN\(^{B}\)

\(^{a}\) Atatürk University, Faculty of Agriculture, Department of Agricultural Biotechnology, 25240-Erzurum, Turkey
\(^{b}\) Atatürk University, Faculty of Agriculture, Department of Plant Protection, 25240-Erzurum, Turkey
\(^{c}\) Ağrı İbrahim Çeçen University, Faculty of Science and Letters, Department of Biology, 04100, Ağrı, Turkey

* Corresponding author. Tel.: +90-442-2311439; Fax: +90-442-2360958; E-mail address: neslidikbas@atauni.edu.tr (N. Dikbas)

Abstract

In this study, a total of 77 *Bacillus cereus* strains, isolated from 4 different food samples (raw milk, chicken, cereal and meat), were tested for their susceptibility against *S. hortensis* essential oil. In addition, it was investigated whether there is any relationship between fatty acid methyl esters of *B. cereus* strains and their susceptibility against *S. hortensis* essential oil. The isolates were clustered into three groups; low susceptible, susceptible and high susceptible. As a results, the percent of susceptibility of *B. cereus* strains isolated from milk, meat, cereal and chicken was found to be 94.83, 75.00, 71.43 and 12.50%, respectively. The high susceptibility (87.50%) and the low susceptibility (5.17%) were observed in *B. cereus* strains isolated from chicken and milk, respectively. Furthermore, the results showed that there is not any relationship between fatty acid methyl esters of *B. cereus* strains and their susceptibility against *S. hortensis* essential oil.

Keywords: Antibacterial, *Bacillus cereus*, essential oil, fatty acid

Introduction

Food preservation has become a complex problem and foodborne diseases are still a major problem in the world [30]. *Bacillus cereus* is a spore-forming bacterium that can be frequently isolated from soil and some foods, and causes two types of food-borne illnesses [15]. Vegetative cells of *B. cereus* are easily inactivated by heating, but spores can survive and after subsequent germination, cause food spoilage [14]. Furthermore, many studies showed that *B. cereus* strains isolated from different food sources have resistant strains against some antibiotics [3; 5; 7; 8; 26]. For this reason, there is a need for new methods of reducing or eliminating food borne pathogens, possibly in combination with existing methods [11].

The increasing incidence of food borne diseases, coupled with the resultant social and economic implications, means there is a constant striving to produce safer food and to develop new natural antimicrobial agents plant essential oils have been used as flavouring agents in food and beverages and, due to the presence of antimicrobial compounds they have
a potential as natural agents for food preservation [13]. A many studies reported a high efficacy of plant essential oils against food-borne pathogens and spoilage bacteria such as *B. cereus, Escherichia coli, Listeria monocytogenes* and *Pseudomonas aeruginosa* etc. [4; 12; 18; 21; 25].

*Satureja hortensis* L. (summer savory) is a well known aromatic and medicinal plant, which is widely distributed in the Eastern Anatolia region of Turkey, and locally named as ‘Koc Otu’. Leaves, flowers and stem of *S. hortensis* are frequently used as tea or additive in commercial spice mixtures for many foods to offer aroma and flavor [10]. Recently, it has been shown that *S. hortensis* extracts and essential oil possess at a broad spectrum of potent antibacterial and antifungal activities [1; 6; 10; 19; 22; 27; 34].

The aim of the present study was to evaluate the relationship between *S. hortensis* L. essential oil susceptibility of *B. cereus* strains isolated from different food sources and their fatty acid methyl ester profiles.

**Materials and Methods**

**Bacterial strains**

In our previous study, a total of 77 *Bacillus cereus* strains had been isolated from the following 4 different food sources: raw milk (58 strains), chicken (8 strains), cereal (7 strains) and meat (4 strains) by using Chromogenic Bacillus Cereus Agar (CM1036) media (Dikbas 2010). It was determined their colony morphology, biochemical and cultural characteristics. Bacteria were grown on NA for routine use, and maintained in Nutrient Broth (NB) with 15% glycerol at -80 °C for long-term storage.

**Plant material**

*Satureja hortensis* L. plants at full flowering stage in july 2009 were collected from Gaziler valley of Senkaya in the Eastern Anatolia region of Turkey. The taxonomic identification of the plant materials was confirmed by a senior plant taxonomist Recep kotan, in the Department of plant protection, Ataturk University, Erzurum, (Turkey). The collected plant material was dried in shade, and the plant leaves were separated from the stems and ground in a grinder with a 2 mm mesh in diameter. The voucher specimen has been deposited in the Biotechnology Research and Application Centre at Ataturk University, Erzurum (Turkey).

**Isolation of the essential oil.**

The air-dried and ground aerial parts of the plants were submitted to water-distillation for 3 h using a Clevenger-type apparatus (yield 1.13% v/w). The essential oil (EO) obtained was dried over anhydrous sodium sulphate and, after filtration, stored in a sealed vial at +4°C until tested and analysed.

**Determination of antibacterial activities**

Antibacterial activity assays were carried out according to (Murray et al. 1995) with a minor modification. The essential oils, carvacrol and thymol dilisions were sterilized by filtration by 0.45 µm Millipore filters. Bacterial suspension (100 µl) containing 1x10⁸ CFU/ml of bacteria spread by a sterile swab on Triptic Soy Agar (TSA) medium. The discs (6 mm in diameter) were impregnated with 12.5 µl of the essential oils (1g/ml dimethylsulfoxide-DMSO) solutions, and put in the middle of the inoculated plates. The bacterial cultures were incubated at 27±2°C for 48 h, and then inhibition zones were measured in diameter (mm) around of the discs. DMSO used as negative control. The assays were performed with three replicates. It was considered to be low susceptible, susceptible and high susceptible strain when the sizes of the inhibition zones were 0-19 mm, 20-29 mm and ≥ 40 mm, respectively.
Determination of the fatty acid methyl esters of bacterial strains by microbial identification system (MIS)

Preparation and analysis of FAMEs from whole cell FA of bacterial strains were performed according to the method described by the manufacturer’s manual (Sherlock Microbial Identification System version 5.5 MIDI, Inc., Newark, DE, USA) (Miller and Berger 1985; Roy 1988). All strains were grown on Difco Trypticase Soy Agar (TSA) for 48 h at 37 °C. Approximately 40 mg cells (wet weight) were transferred to 13x100 mm glass tubes fitted with Teflon-lined screw caps and fatty acids were extracted using methods described by Miller (1982). FAMEs were separated by gas chromatography (HP6890, Hewlett Packard, Palo Alto, CA, USA) with a fused-silica capillary column (25 m x 0.2 mm) with cross-linked 5% phenyl methyl silicone. FAME profiles of each bacterial strain were identified by comparing the commercial databases (TSBA 40) with the MIS software package.

Statistical analyses of FAMEs

In order to determine whether there is a statistically significant difference among the obtained FAMEs for 3 clusters (resistant, susceptible and high susceptible strains), variance analyses were carried out using SPSS 10.0 software package. Differences between means were tested by Duncan test (p≤0.05).

Table 1. Antibacterial activity of the essential oil isolated from Satureja hortensis against Bacillus cereus strains

<table>
<thead>
<tr>
<th>Origin</th>
<th>NTI</th>
<th>LSus 1-19 mm</th>
<th>%</th>
<th>Sus 20-39 mm</th>
<th>%</th>
<th>HSus 40-49 mm</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>58</td>
<td>0</td>
<td>0.00</td>
<td>55</td>
<td>94.83</td>
<td>3</td>
<td>5.17</td>
</tr>
<tr>
<td>Meat</td>
<td>4</td>
<td>0</td>
<td>0.00</td>
<td>3</td>
<td>75.00</td>
<td>1</td>
<td>25.00</td>
</tr>
<tr>
<td>Chicken</td>
<td>8</td>
<td>0</td>
<td>0.00</td>
<td>1</td>
<td>12.50</td>
<td>7</td>
<td>87.5</td>
</tr>
<tr>
<td>Cereal</td>
<td>7</td>
<td>0</td>
<td>0.00</td>
<td>5</td>
<td>71.43</td>
<td>2</td>
<td>28.57</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>0</td>
<td>0.00</td>
<td>64</td>
<td>83.11</td>
<td>13</td>
<td>16.89</td>
</tr>
</tbody>
</table>

NTI: The number of total isolates; LSus: Low susceptible; Sus: Susceptible; HSus: High susceptible

Results and Discussion

Due to its S. hortensis antimicrobial and antioxidant potential is becoming increasingly important in food studies [1; 12] Many publications have previously documented the high antimicrobial activity of the essential oil or extracts of S. hortensis [2; 6; 10; 19;22;31;33;36]. Our result is in agreement with the literature reports on the essential oils of S. hortensis.

Antimicrobial activity test results were given in Table 1. The results indicated that all of the strains were susceptible or highly susceptible against S. hortensis essential oil. The percentage of the susceptible and highly susceptible strains was 83.11% and 16.89%, respectively. None of the strains were resistant against tested essential oil. In our previous study, it was tested for the effectiveness of penicillin on the B. cereus strains [26]. According to the results, all of the strains isolated from milk, meat, chicken and cereal were resistant against penicillin (Table 2). The current study showed that the effectiveness of S.hortensis essential oil was found to be more powerful than that of penicillin. The percent of susceptibility of B. cereus strains isolated from milk, meat, cereal and chicken was found to be 94.83, 75.00, 71.43 and 12.50%, respectively. The high susceptibility (87.50%) was observed in B. cereus strains isolated from chicken. The low percent of susceptibility was observed at B. cereus strains from milk as 5.17%. Güllüce et al. (2003) stressed that thymol (29.0%), carvacrol (26.5%), μ-terpinene (22.6%) and p-cymene (9.3%) were the main
components of the *S. hortensis*. These compounds can be responsible for the antibacterial activity of *S. hortensis* oil. Further studies including other important food pathogens, such as *Listeria monocytogenes*, *Clostridium perfringens*, *Clostridium botulinum*, *Bacillus cereus*, *Vibrio parahaemolyticus*, *Campylobacter jejuni*, are needed to improve our understanding of the influence of *S. hortensis* on food born bacteria and fungi.

Table 2. Antibacterial activity of the penicillin antibiotic against *Bacillus cereus* strains (Dikbas 2010)

<table>
<thead>
<tr>
<th>Origin</th>
<th>NTI</th>
<th>LSus 1-19 mm</th>
<th>LSus 20-39 mm</th>
<th>LSus 40-49 mm</th>
<th>Sus 1-19 mm</th>
<th>Sus 20-39 mm</th>
<th>Sus 40-49 mm</th>
<th>HSus 1-19 mm</th>
<th>HSus 20-39 mm</th>
<th>HSus 40-49 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>58</td>
<td>58</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Meat</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Chicken</td>
<td>8</td>
<td>8</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cereal</td>
<td>7</td>
<td>7</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

NTI: The number of total isolates; LSus: Low susceptible; Sus: Susceptible; HSus: High susceptible

Based on the results of susceptibility of the strains against the essential oil, the isolates were clustered into three groups as resistant, susceptible and high susceptible. As shown in Table 3, a total of 12 different fatty acids were detected in 77 strains tested but 6 of them appeared as minor component, in less than %5. The strains had 15:0 iso 3OH (29.86%), 16:0 iso (11.20%), 17:0 iso (9.18%), 13:0 iso (8.66%), 16:0 (9.15%) and 14:0 iso (6.66%) as major components. It is well known that variation in growth conditions can affect lipid composition in bacteria. Güven and Mutlu (2009) also found the same results of this study. However, another study showed that FAs iso 17:1 ω10c is present in *B. cereus* vegetative cells and spores, and 16:0 2OH and 17:0 iso 3OH are present in spores but not in the vegetative cells [28].

Table 3. Relationship between the fatty acid methyl esters (FAMEs %) and essential oil susceptibility of *Bacillus cereus* strains

<table>
<thead>
<tr>
<th>FAMEs</th>
<th>LSus 1-19 mm</th>
<th>LSus 20-39 mm</th>
<th>LSus 40-49 mm</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>15:0 iso 3OH</td>
<td>30.22</td>
<td>28.37</td>
<td>29.02</td>
<td>29.86±5.95</td>
</tr>
<tr>
<td>16:0 iso</td>
<td>10.94</td>
<td>12.31</td>
<td>11.76</td>
<td>11.20±2.76</td>
</tr>
<tr>
<td>16:0</td>
<td>8.72 a</td>
<td>11.26 b</td>
<td>10.04 ab</td>
<td>9.18±2.82</td>
</tr>
<tr>
<td>13:0 iso</td>
<td>8.90</td>
<td>7.73</td>
<td>8.07</td>
<td>8.66±3.75</td>
</tr>
<tr>
<td>17:0 iso</td>
<td>9.09</td>
<td>8.08</td>
<td>9.79</td>
<td>9.15±3.47</td>
</tr>
<tr>
<td>14:0 iso</td>
<td>6.70</td>
<td>6.44</td>
<td>6.62</td>
<td>6.66±1.90</td>
</tr>
<tr>
<td>14:0</td>
<td>4.56</td>
<td>4.51</td>
<td>4.47</td>
<td>4.54±1.41</td>
</tr>
<tr>
<td>15:0 anteiso</td>
<td>4.71</td>
<td>5.66</td>
<td>4.39</td>
<td>4.78±2.09</td>
</tr>
<tr>
<td>16:1 ω6c</td>
<td>3.89</td>
<td>3.99</td>
<td>3.84</td>
<td>3.89±1.54</td>
</tr>
<tr>
<td>16:1 ω7c</td>
<td>3.89</td>
<td>3.99</td>
<td>3.64</td>
<td>3.89±1.54</td>
</tr>
<tr>
<td>17:0 anteiso</td>
<td>1.47a</td>
<td>2.64b</td>
<td>1.87ab</td>
<td>1.65±1.56</td>
</tr>
<tr>
<td>12:0 aldehyde</td>
<td>1.27ab</td>
<td>0.32a</td>
<td>1.65b</td>
<td>1.21±1.50</td>
</tr>
<tr>
<td>Others</td>
<td>5.64</td>
<td>4.70</td>
<td>4.84</td>
<td>5.33</td>
</tr>
</tbody>
</table>

LSus: Low susceptible; Sus: Susceptible; HSus: High susceptible

SEM: Means ± standard error in the same line by the same letter is not significantly different according to the test of Duncan (P≤ 0.05)

15:0 iso 3OH, 13:0 iso, 14:0 iso and 14:0 fatty acids were absent the highest rate in low susceptible strains than that of the susceptible and high susceptible strains. But, these differences were not statistically significant. On the other hand, 16:0 iso, 16:0, 17:0 anteiso
and 12:0 aldehyde fatty acids in the low susceptible were lower than that of the susceptible and highly susceptible strains. Differences of the percentages of 16:0 and 17:0 anteiso fatty acids between the low susceptible and susceptible strains were not significant statistically. The percentages of 14:0, 15:0 anteiso, 16:1 \( \omega 6c \) and 16:1 \( \omega 7c \) fatty acids in the high susceptible strains were less than that of low susceptible and susceptible strains. Differences of the percentages of them were not significant statistically. Many studies stated that FA analysis is a useful tool for identifying bacterial species including \textit{Bacillus} [9; 28; 32]. In a previous study, we also reported a FAME analysis procedure which was successfully employed for the identification of \textit{B. creus} strains [26].

\textit{S. hortensis} can be added as a protective agent to various food products. A food product requires a very low initial microbial load and inhibition during the production period for an adequate shelf-life. Some natural essential oils assayed were highly inhibitory to a selected pathogen (\textit{B. cereus}), and they might provide alternative technologies to conventional antimicrobial additives in foods. As a result, several aspects need to be studies before adding plant essential oils into food, such as their toxicity, their allergenicity, the effect of processing conditions on their efficiency and the concentration of oil required in foods.

To our knowledge, there is a lack of information on the susceptibility of the same bacterial strains against plant essential oil or extracts. In this study showed that there is not any relationship between fatty acid methyl esters of \textit{B. cereus} strains and their susceptibility against \textit{S. hortensis} essential oil. Some natural essential oils (\textit{S. hortensis} etc.) assayed were highly inhibitory to a selected pathogen (\textit{B. cereus}), and they might provide alternative technologies to conventional antimicrobial additives in foods. Further studies are needed to investigate the oils incorporation into appropriate food formulation, and evaluate flavor, chemical changes and antimicrobial effect in the whole food system.

References


