

Anti-adhesion activity of mint (*Mentha piperita* L.) leaves extract against beverage spoilage bacteria *Asaia* spp.

Hubert Antolak,* Agata Czyżowska, Dorota Kregiel

Institute of Fermentation Technology and Microbiology,
Lodz University of Technology, Wolczanska 171/173, 90-924 Lodz, Poland

*hubert.antolak@gmail.com

Abstract: *The production of a functional beverage, supplemented with fruit flavourings meets the problem of microbiological contamination. The most frequent source of such spoilage is the bacteria from the relatively newly discovered genus Asaia. It causes changes in organoleptic properties, creating turbidity, haziness and distinctive sour odour as well as biofilm on production lines which are responsible for secondary contamination of products. For this reason, new methods using natural preservatives are being developed to minimize this microbiological contamination. The application of some plant extracts as an additives in functional beverages production is presumed to have a beneficial effect on reducing adhesive abilities of the bacteria. The aim of this research was to investigate the effects of mint leaves extract on the Asaia lannensis and Asaia bogorensis adhesive abilities to polystyrene. The bacterial adhesion was analysed by means of plate count method and luminometric tests. Additionally, plant extract was subjected to high performance liquid chromatography (HPLC) analysis, in order to check polyphenols content. Results indicates that 10% (v/v) addition of mint extract significantly reduced Asaia spp. adhesion to polystyrene. Due to the presence of bioactive compounds in the used extract, it can be used as an additive to increase microbiological stability as well as health promoting values of beverages.*

Keywords: *Asaia* spp., mint extract, anti-adhesive properties, functional beverages.

Introduction

Functional drinks are products, which beyond the primary task – thirst quenching, attributed to pro-health effect on the human body. Functional beverages, are a source of vitamins, minerals and organic acids, necessary for the proper functioning of human body [1]. However, these compounds may also provide additional nutrients for spoilage microorganisms. The addition of common preservatives (benzoate, sorbate or dimethyl dicarbonate) and a low pH does not inhibit the growth of acidophilic bacteria and yeasts. Additionally, in

certain conditions of pH and temperature stabilizers and preservatives may be decomposed to other compounds suitable to growth of microorganisms [2]. The use of additives with questionable quality, changes in technology processing or packaging may expose producers of functional beverages to an increased risk of microbial spoilage, including genera or species previously unknown as food contaminants [2].

Numerous studies had recently shown that common microbial spoilage in functional drinks are acetic acid bacteria *Asaia* spp. The growth of these bacteria results in organoleptic changes of final product, formation of haziness and flocculation [3]. What is more, the bacteria show the adhesion ability to various abiotic surfaces such as glass, polypropylene, polystyrene, commonly used as packaging or installation materials in food industry [4]. Biofilms formed on inner surfaces of production lines can cause changes in fluid turbulence, especially at bending pipes, valves or filters. Consequently, it can cause failures of equipment and secondary contamination of final products [3]. The proliferation and surface's colonization by *Asaia* spp. cells is a serious problem in beverage industry. That is why, increasing attention on natural agents in the biofilm inhibition is observed. These strategies use natural bioactive compounds, e.g. polyphenols, saponins or organic acids. The documented positive effects of phenolic compounds as anti-cancer, anti-cardiovascular agents may be extended to anti-microbial and anti-adhesive actions [5].

Therefore, in our work, we used a specific natural extract obtained from the leaves of peppermint, a plant commonly found in Poland, as an anti-microbial and anti-adhesion agent against bacteria *Asaia* spp.

Experimental

Materials

Plant extract

After harvesting, herbs were washed and gently dried on paper towels and then tied in loose bunches to ensure good air circulation. In order to protect them from dust and contamination, the herbs were put into paper bags. Small punch holes in the bags allowed for good ventilation. Drying was carried out in a dark room at 30°C for 30 days. Then, leaves, separated from the stems, were crushed using a laboratory mortar and 50 g were then placed in 500 cm³ dark glass bottles which were filled with 250 cm³ of 10% (v/v) ethanol. These were stored in room temperature for one month, with occasional agitation for more efficient extraction of the polyphenolic compounds [6]. Subsequently, the macerated extract was centrifuged at 6500 rpm for 15 minutes at 20°C (Eppendorf). Peppered plant extract was added to the culture media to a final concentration of 10% (v/v). The liquid culture media with plant extracts were filtered and sterilized using microfiltration with 0.45- μ m-pore-size membranes (Millipore).

Bacterial strains

In this research, strongly adhesive bacteria *Asaia bogorensis* ISD1 (GenBank KP234014), *Asaia bogorensis* ISD2 (GenBank KP234015), *Asaia bogorensis* FFMW (GenBank KC756841), *Asaia lannensis* IFMW (GenBank KP234011), *Asaia lannensis* IFCW (GenBank KP234012) and *Asaia lannensis* FMW1 (GenBank HQ917850) were used.

Culture conditions

The adhesion was examined in liquid culture media: minimal medium [0.3% (NH₄)₂PO₄ (w/v), 0.3% KH₂PO₄ (w/v), 0.3% MgSO₄×7 H₂O (w/v), 0.05% (w/v) yeast extract] and flavored mineral water [8.1% (w/v) sucrose, 0.05% (w/v) strawberry flavor, 0.16% (w/v) citric acid, 0.02% (w/v) sodium benzoate, 0.02% (w/v) velcorin].

The 20 cm³ of sterile media were poured aseptically into 25 cm³ Erlenmeyer flasks covered with a textile cloth in order to ensure aerobic conditions. Sterile polystyrene carriers were placed vertically in a liquid culture medium in such a way that half of the carrier was immersed in the medium, and the other part was above the liquid. The media were inoculated with standardized bacterial suspensions, to obtain final cells concentration in the medium was approximately 10⁵- 10⁶ CFU/cm³ at the beginning of the experiment.

The culture were incubated at 25°C for 6 days on a laboratory shaker adjusted to 130 rpm. The adhesion determination for each bacterial strain was carried out after 0th, 3rd and 6th day of incubation.

Adhesion surface

Asaia spp. adhesion was carried out on the polystyrene (PS) (Coveris Rigid Poland, Skierniewice) slides measuring 76×26 mm. This material is certified by Polish National Institute of Public Health and approved for contact with food.

Methods

Adhesion control

The adhesion analysis was performed by the plate count method and luminometric measurements described by Kregiel (2013) [8]. In order to determine the number of the bacteria attached to polystyrene surface, the carrier was removed from the medium and swabbed with a sterile contact swab. Subsequently, the removed bacterial biofilm was placed in a saline solution with 0.1% (w/v) Tween 80, vortexed and the appropriate dilutions were prepared. The dilutions were then transferred onto a GC agar medium [2% (w/v) glucose, 0.3% (w/v) yeast extract, 0.3 % (w/v) peptone, 0.7 % (w/v) CaCO₃, 2 % (w/v) agar] and incubated for 96 h at 25°C. After incubation, the colonies of *Asaia* spp. were counted and the colony forming units per square centimetre (CFU/cm²) value was determined. Analogous procedure and incubation conditions were used to determine the number of bacteria in the culture medium. Results were given as colony forming units per cubic centimetre (CFU/cm³). From the obtained values,

relative adhesion coefficient A(%) was calculated using the formula $A(\%) = (N_a/N_p) \times 100\%$, where N_a is the number of attached cells to a carrier and N_p is the number of planktonic cells in the culture medium.

For luminometric analysis, the carriers were removed from the media, washed with sterile distilled water and swabbed with pens for ATP sampling (Merck). Measurements were made using a HY-LiTE® 2 luminometer (Merck). The results were converted into the Relative Light Units per square centimetre (CFU/cm²).

Chemical constituents analysis

Phenolic compounds in the plant extract were determined using high performance liquid chromatography (HPLC) method described by Antolak et al. (2015) [9]. Polyphenolic profile was determined using HPLC-DAD method with a diode array detector (Finnigan Surveyor-PDA Plus detector), and a Chrom Quest 5.0 chromatography software (Thermo Fisher Scientific Inc, Waltham, MA, USA). Separation was achieved on a Lichrospher RP 18-5 (Hichrom, Berkshire, UK) (250×4.6 mm, 5 µm packing). Detection was provided on 280 and 320 nm.

Statistics

Means with standard deviations were calculated from the data obtained from three independent experiments. The mean values of the adhesion results were compared using one-way repeated measures analysis of variance (ANOVA; OriginPro 8.1, OriginLab Corp., Northampton, MA). Statistical significance was set at the conventional level of 5% ($P < 0.05$).

Results and Discussion

Analysis of adhesion by *Asaia* spp. strains to polystyrene surfaces was carried out in minimal medium, strawberry mineral water, as well as in media with 10% (v/v) mint extract. The results of studies using plate count method and luminometry, reported as adhesion coefficient A (%) and adhesion (RLU/cm²), respectively are presented in Figures 1 and 2.

Both methods showed that commercial flavour mineral water is a suitable environment in which the process of adhesion and biofilm formation occurs efficiently. The relative coefficient A(%) for minimal medium ranged from 0.4% for *Asaia lannensis* IFMW to 1.1% for *A. lannensis* IFCW, while in the strawberry water these stains reached the lowest values (0.2%). On the other hand, the stronger adhesion in flavoured water were observed for *A. bogorensis* strains of which ISD1 reached the highest coefficient value (3.4%). These results were confirmed by luminometric analysis. In minimal medium adhesion ranged from 2850 RLU/cm² for *Asaia lannensis* FMW1 to 10 016 RLU/cm² for *Asaia bogorensis* ISD2. On the other hand, the highest RLU for biofouling in strawberry water were noted for *A. bogorensis* ISD2 (10 080 RLU/cm²). The results agree with those obtained by Kregiel (2013), Kregiel et al. (2014) and Antolak et al. (2015). The research showed that culture medium composition is an important parameter for *Asaia* spp. adhesion [7, 8, 9]. This is confirmed by our

research, where *A. bogorensis*, in particular, showed a stronger adhesive abilities in commercial flavour mineral water. Beside the kind of strain, nature of environment and carbon source, the type of adhesion surface, especially surface hydrophobicity, has important role in stimulating the biofouling processes. It is known that materials with low surface free energy constitute a more favorable surfaces for microbial cells to colonize. Plastic materials commonly used in beverage industry have low free energy values, e.g. for polyethylene terephthalate (PET) this parameter equals 44 mN/m at 20°C while for polystyrene (PS) it is approximately 40 mN/m at 20°C. For hydrophilic glass surfaces this value is significantly higher (70 mN/m at 20°C) [10]. Thus, the plastic materials commonly used in the food industry, characterized by low energy, stimulate bacterial adhesion and biofilm formation [11].

The addition of mint leaves extract caused a significant reduction in the adhesion of almost all *Asaia* spp. strains. The significant decrease in luminometric results was observed for *Asaia bogorensis* ISD1 and *A. bogorensis* ISD2. For these two strains, incubated in commercial strawberry water, these values were reduced from the 8640 RLU/cm² to 944 RLU/cm² and from 10 080 RLU/cm² to 1136 RLU/cm², respectively. Significant differences ($p < 0.05$) were also reported in the case of the adhesion coefficient A(%). The meaningful decrease of this parameter was noted for *Asaia bogorensis* ISD1 (from 3.4% to 0.15%) and *A. bogorensis* ISD2 (from 1.4% to 1.3%) strains. It was noted that mint extract shows anti-microbial effect against wide spectrum of human pathogens. The anti-microbial activity of menthol and menthone from mint leaves was confirmed against bacteria *Klebsiella pneumoniae*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staph. mutans* as well as yeast *Candida albicans* [12, 13]. However, literature data on anti-adhesive and anti-biofilm activities of mint extracts is limited. Previous reports showed that essential oils and extracts from *Mentha piperita* show inhibition of biofilm formation by Gram negative rods *Pseudomonasa aeruginosa*, Gram positive bacilli *Listeria monocytogenes*, and yeasts *Candida albicans*, *C. dubliniensis* [14, 15, 16]. Sandasi and co-workers noticed significant decrease in the number of attached cells of *Pseudomonas aeruginosa* in the presence of mint extract. In the same work the authors noted that mint extract inhibits adhesion and biofilm formation of *Candida albicans* [15]. It is believed that the anti-adhesive properties may be a result of synergistic actions of bioactive compounds from extracts of *M. piperita* [17].

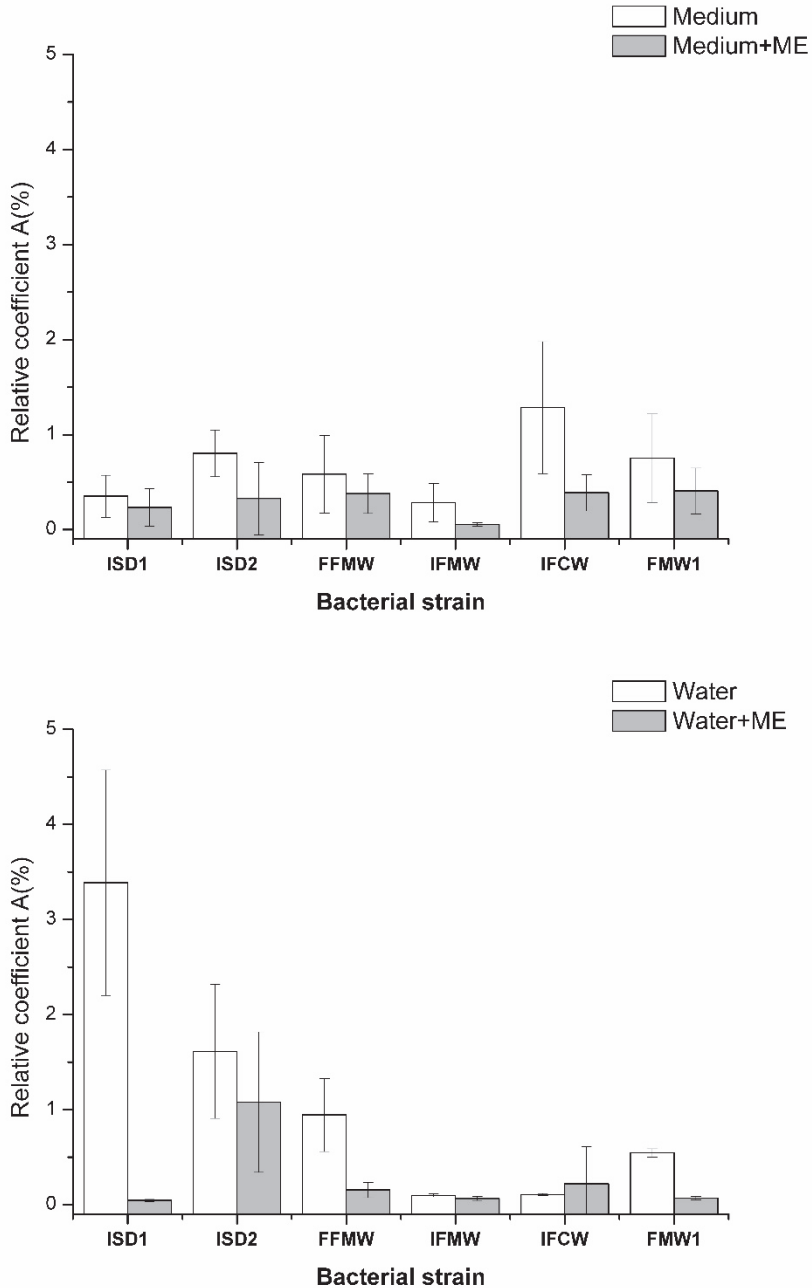


Figure 1. Relative adhesion coefficient A(%) in the minimal medium (Medium) and strawberry water (Water) with mint extract (+ME). Control samples were the same culture media but without mint

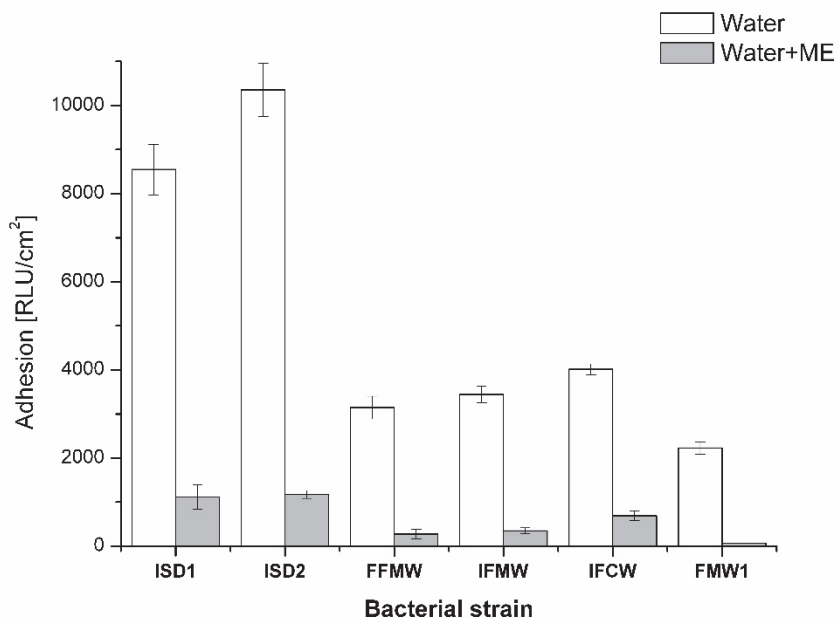
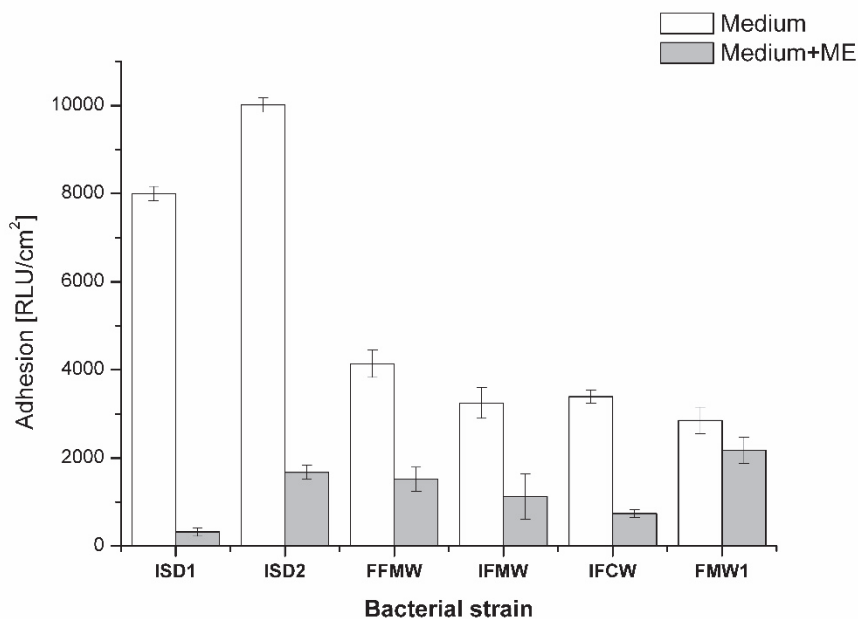


Figure 2. Adhesion (RLU/cm²) in the minimal medium (Medium) and strawberry water (Water) with mint extract (+ME). Control samples were the same culture media but without mint

As it can be seen in Table 1, the mint extracts used in the study were a source of phenolic compounds, such as gallic, chlorogenic, neochlorogenic, caffeic, coumaric and rosmarinic acids. Peppermint is well known as a remedy for coughs, bronchitis, inflammation of the oral mucosa and throat. The phenolic acids content in mint extracts influence on the biological activities of this plant material. It was documented that ferulic acid showed anti-inflammatory and anti-tumoral activities, while gallic acid showed cytotoxicity against tumor cells and induced apoptosis in numerous cancer cell lines [18]. What is more, some phenolic acids such as gallic and caffeic showed good activity against the Gram negative rods *K. pneumoniae* and Gram positive cocci *Staph. epidermidis* and *Staph. aureus* [19]. Thus, it is possible that the anti-microbial action of mint extract is the result of activity of essential oils (menthol, menthone, menthyl acetate and menthofuran as well as phenolic acids [17].

Table 1. Main phenolic compounds in mint extract

No.:	Time [min]	λ_{\max} [nm]	Proposed molecule
1	4.81	220, 270	Gallic acid
2	11.07	324	Neochlorogenic acid
3	17.39	325	Chlorogenic acid
4	18.84	321	Caffeic acid
5	22.83	293sh, 309	<i>p</i> -Coumaric acid
6	30.95	254, 290, 328	Rosmarinic acid
7	33.99	322	Caffeic acid derivative

The outcomes of adhesion studies indicated that the intensity of bacterial attachment to polystyrene material depends on the chemical characteristic of the environment. Strawberry water created more favorable conditions to form biofilm for *Asaia* spp. in comparison to the minimal medium. Our study shows that *M. piperita* extracts inhibit adhesion of both *A. lannensis* and *A. bogorensis* strains. Therefore, peppermint may prove to be promising candidates not only as health-promoting, functional additives but also as a natural, antiadhesive agents in soft drinks.

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