# Growth of *Asaia* spp. in Flavored Mineral Water - Evaluation of the Volumetric "Bottle Effect"

Dorota Kręgiel* and Hubert Antolak

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

**Abstract:** This study evaluates the growth of acetic acid bacteria *Asaia* spp. in fruit flavored mineral water in both plastic and glass bottles with different volumes. Two strains of bacteria *A. bogorensis* and *A. lannensis* were isolated from spoiled soft drinks. Bacterial growth was analyzed using the plate count method and light microscopy. The results indicate that the tested strains of *Asaia* spp. exhibit the better growth in the polytereftalate bottles with small volumes: 0.33 L and 0.5 L. The microscopic observations documented that these bacteria are able to form visible cell co-aggregates after 2-week period storage at 25°C. Using glass as packaging material allows for the slower growth of *Asaia* spp. and contributes to improving the microbiological stability of fruit-flavored mineral waters.

**Keywords:** *Asaia* spp., Growth, Flavored water, Packaging materials, Bottle effect.

## INTRODUCTION

Beverage industry is probably one of the fastest and most dynamically developing branches of the food industry. Bottled functional beverages and flavored waters are gaining popularity [1]. This follows from the concept of 'wellness' - an increasing awareness of physical well-being and good health of the consumers. Consequently, a broad range of products is produced to fit the wellness mould: from bottled water, juice-based and tea-based drinks to the soft drinks containing wild spectrum of the ingredients to providing an additional functional benefit [2]. Fulfilling growing demand from consumers for natural, fresh and tasty products, leads to an increase in the content of juice, reduce the level of acidity and reduce or even eliminate heat treatment processes or content of artificial preservatives. As a result of the development of international trade, changes in the composition of products, technology, processing or packaging antimicrobial barrier, present so far in the traditional soft drinks, have ceased to exist [3]. These facts may expose producers from beverage industry to an increased risk of microbiological contamination, including microbes previously unknown in the production environment [4]. The example of bacteria, newly isolated as a contamination of a functional beverages are gram-negative rods belonging to the genus *Asaia*. It is documented that a source of these bacteria are fruit concentrates used for beverage production. These bacteria were characterized by strong adhesion properties to the packaging materials, such as polystyrene and polyethylene terephthalate [5, 6]. Biofilm-forming ability may be responsible for secondary contamination of final products [7].

The risk of microbial contamination of soft drinks is associated with the type product, its composition as well as the packaging material and the bottling manner. Microbial batch growth during incubation in bottles of various sizes and volumes is the subject to the so-called effect "bottle effect" or "volume effect". The particular section of "bottle effect" literature focuses specifically on a volumetric "bottle effect" [8]. One of the oldest and best-known studies summarized clearly: "It will be observed that the densest bacterial populations appear in the bottles of water which offer the largest area of glass surface per unit volume of water". Bischofberger *et al*. [9] observed that groundwater incubation led to significantly more growth (about 2 log units) in small bottles (100 ml) than in big ones (10 liters).

There are two main types of packagings used in the beverage industry, historically older glass, and plastic materials, *e.g*. polyethylene terephthalate (PET). Glass is still the preferred packaging material for high quality fruit beverages, although the hot-fill/hold/cool process must be applied with care, in order to avoid container breakage. The plastic materials are inexpensive, flexible, durable, and chemically resistant. However, the oxygen content in plastic bottles increases with time, whereas glass bottles are impermeable to oxygen [10].

In the present study, due to the increasing number of the reported cases of *Asaia* spp. spoilage in soft

*Address correspondence to this author at the Institute of Fermentation Technology and Microbiology, Lodz University of Technology, Wólczańska 171/173, 90-924 Łódź, Poland; Tel: +48426313247; Fax: +48426365976; E-mail: dorota.kregiel@p.lodz.pl

E-ISSN: 2408-9826/16 

drinks, we took a simple but detailed look at the effect of bottle size on the long-term batch growth of *Asaia* species in still flavored mineral water.

## MATERIALS AND METHODS

### Biological Material

The study used two strains belonging to the genus *Asaia,* isolated from spoiled fruit-flavored water: *A. bogorensis* strain FFMW and *A. lannensis* strain FMW1. These bacteria were identified using morphological, physiological and genetic methods and the nucleotide sequences have been reported to the base of the National Center for Biotechnology Information (NCBI GenBank) with the accession numbers: KC756841 and HQ917850 respectively [7, 10].

### Culture Conditions

The growth of *Asaia* strains was monitored in unit packagings made from glass (G) and polyethylene terephthalate (PET). The bottle volumes were as follows: for PET bottles - 0.25 L, 0.33 L, 0.5 L, 0.7 L, and for glass ones - 0.25 L, 0.33 L, 0.45 L, 0.7 L. In the experiments, a sample of commercial fruit-flavoured mineral water (8.1% saccharose (w/v), 0.05% strawberry flavor (w/v), 0.16% citric acid (w/v), 0.02% sodium benzoate (w/v), 0.02% velcorin (w/v)) was filter-sterilized with a 0.45-μm-pore-size membrane, bottled and inoculated to obtain about $10^5$ cells/mL in the start of experiment. All plastic caps for bottles were sterilized by 70% v/v ethanol treatment, washed sterile distilled water and dried inside the laminar flow cabinet (Telstar Bio-II-A/M). Prepared bottled samples of inoculated flavored water were stored at 25°C ± 5°C for 9 weeks.

### Growth Analysis

The analysis of *Asaia* spp. growth was performed after 3, 6 and 9 weeks of incubation. Each time, the macroscopic and microscopic observations (a light microscope OLYMPUS BX41 with a digital camera DP72) assessing turbidity and co-aggregation of bacterial cells in inoculated samples of bottled flavored water were was carried out. Simultaneously, the number of bacterial culturable cells was determined using plate count method and GC agar medium (2% glucose (w/v), 0.3% peptone (w/v), 0.3% yeast extract (w/v), 0.7% $CaCO_3$ (w/v)). Inoculated plates were incubated at 25°C for 5 days, and the characteristic, pink colonies with clear zone around each colony, were counted. The results were presented as colony forming units per mL of flavored water (CFU/mL).

### Statistic

Mean values were calculated from the data obtained from three independent samples, and the standard deviations (SD) were calculated. The means of the growth results were compared using one-way repeated measures analysis of variance (ANOVA; Origin 8.1, Origin Lab Corp., Northampton, MA). Statistical significance was set at the conventional level of 5% ($P<0.05$).

## RESULTS

The macroscopic and microscopic observations of bottled flavored water evidenced that both growth and co-aggregation of bacterial cells were evident in PET bottles after 2-3 weeks in comparison to glass bottles where the growth of bacteria was faintly visible after 8 weeks. Additionally, it was noted that these processes were also dependent on the volume of PET bottles. The evidence of bacterial contamination were seen earlier in PET bottles with a capacity of of 0.33 L and 0.5 L. In addition, the microscopic observations showed that in PET bottles the *Asaia* spp. were able to form more compact, large cell aggregates - "flocks"(Figure **1**). The results of microbiological analysis of bacterial

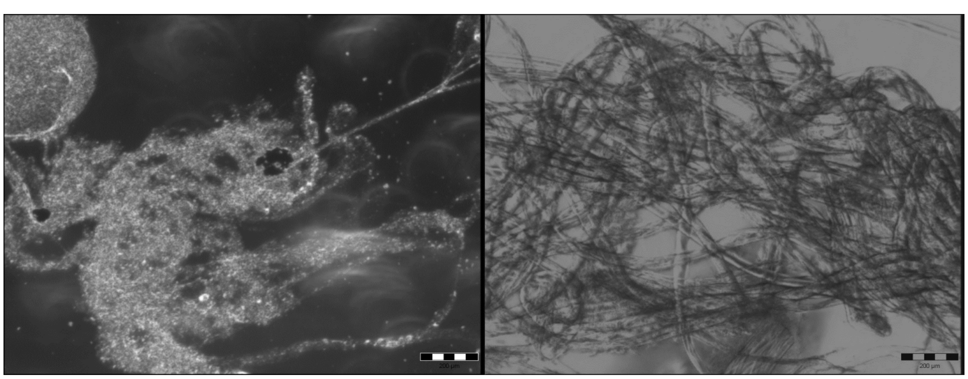

**Figure 1:** Growth of *A. lannensis* (left side) and *A bogorensis* (right side) in "flock" forms. Microscopic observations.

growth indicates that both *Asaia* spp. strains achieved the significantly higher ($p<0.05$) number of culturable cells in plastic bottles in comparison to glass packagings (Figure **2**). The highest number of *A. lannensis* cells ($1.3 \pm 0.3 \times 10^8$ CFU/mL) was noted after 9 weeks of storage in 0.33L PET bottles, while for *A. bogorensis* strain the highest result ($3.4 \pm 0.3 \times 10^8$ CFU/mL) was noted for 0.5L PET packagings. Respectively, for glass bottles the highest growth of *A. lannensis* ($3.8 \pm 0.6 \times 10^7$ CFU/mL) and *A. bogorensis* ($5.7 \pm 0.5 \times 10^7$ CFU/mL) was observed in the 0.45L bottles. For almost all 0.7L containers with *A. bogorensis* we noted a decrease in bacterial population. However, this trend was not observed for the strain *A. lannensis*. The results obtained for the 9 week-incubation indicates that *A. bogorensis* showed better growth in flavored water than *A. lannensis* (Figure **3**). Especially visible differences were noted for the bacterial cultures in PET bottles of 0.33 L and 0.5 L.

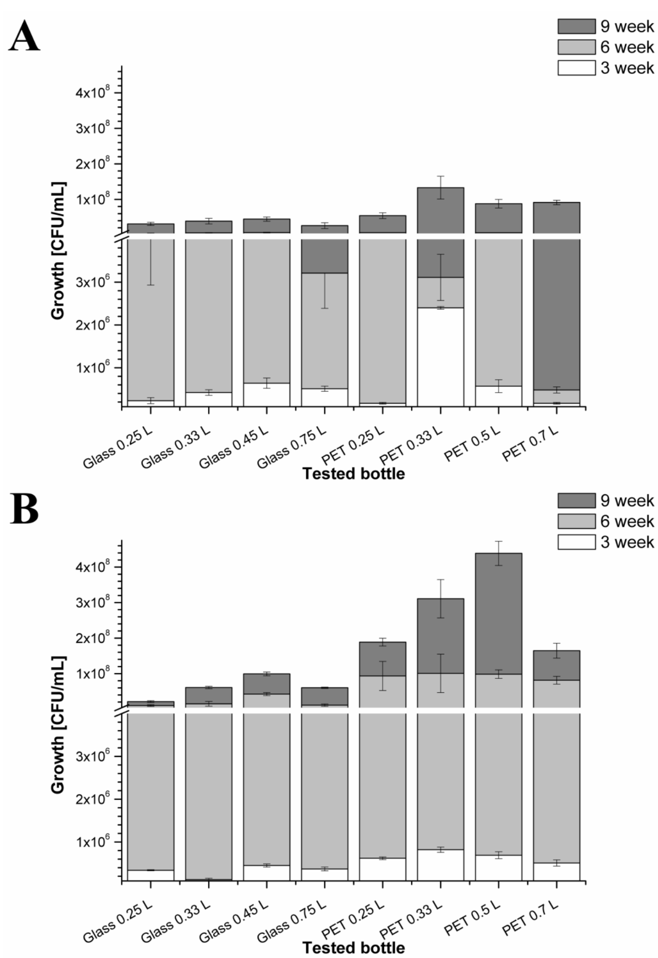


**Figure 2:** Growth of *A. lannensis* (**A**) and *A. bogorensis* (**B**) in fruit flavored mineral water packaged in the glass and PET bottles with different volumes.

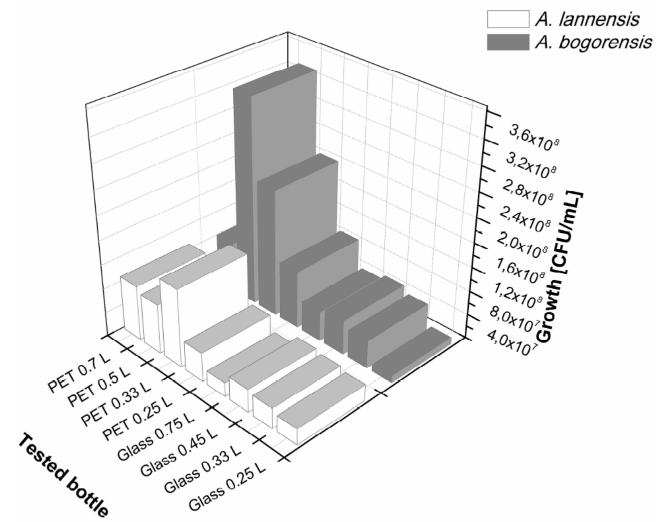


**Figure 3:** Comparison of *A. lannensis* and *A. bogorensis* growth in flavored mineral water, packaged in glass and plastic bottles with different volumes, after 9-week storage.

## DISCUSSION

The fact that microbiological processes during confined incubation differ and depend on the environment is undeniable. The particular section of "bottle effect" literature focuses specifically on a volumetric "bottle effect", where the growth effects are linked specifically with the size (or surface-to-volume ratio) of the incubation vessel [8]. The results are rather controversial. For example, Hammes and co-workers did not observe evidence of a volumetric bottle effect on short-term (few-days) batch incubations, and other authors observed results implying apparent bottle effects during growth studies [8, 9]. In this context, our studies describing the growth of spoilage bacteria *Asaia* spp. in different types of packagings and volumes are the first.

Bacteria *Asaia* spp. are often isolated from flavored mineral waters [7, 11, 12] although their natural habitat is tropical flowers and insects. It was shown that these bacteria are able to form cell aggregates [5]. Our studies confirmed that *Asaia* spp. aggregates are formed in spoiled flavored water after 2 weeks of storage, and these structural forms are strain dependent and various from spongy to fibrous. Best growth of *Asaia* spp. strains was noted in small PET bottles from 0.33 L to 0.5 L. In our study two different species belonging to one genera *Asaia* were used, and the observed "bottle effect" was strain – depended. According to the literature, even within one species, strains can differ by the growth, oxygen/nutrient requirement, and many other characteristics [9]. Our results confirmed the observations Bischofberger *et al*. [10] relating to "bottle efeect" for groundwater. This phenomenon may be explained by the fact that smaller bottles provide a larger contact area with the surface of the liquid medium bottle [8, 10]. However, "bottle effect can also depend on the type of packaging material.

The use of plastics in beverage packaging is developed due to low cost of materials and functional advantages: thermosealability, microwavability, optical properties, and unlimited sizes and shapes. In addition, plastic materials can be manufactured either as a single film or as a combination of more than one plastic by lamination or co-extrusion. Combining materials results in the additive advantage of properties from each individual material and often reduces the total amount of packaging material required [13]. However, in contrast to traditional packaging material - glass, bottles made with plastic are permeable at different degrees to small molecules, like gases, water or organic vapour, and to other low molecular compounds like aromas, flavors and other food additives [14]. Additionally, beverages packaged in plastic bottles are particularly exposed to risk of microbial growth, as the oxygen content increases during storage [15].

## CONCLUSION

Our studies are related to beverage packaging in different materials; focusing the attention on the bacterial growth over time. The obtained results indicate that both the type of packaging material and its volume may influence on *Asaia* spp. growth, however, this effect was also strain – depended. Generally, the best choice in terms of microbiological stability of final products tend to be glass containers with volume more than 0.7 L. Study on "bottle effect" in flavored mineral waters and other functional drinks seems to be very interesting not only from a scientific point of view. It is also necessary to help beverage industry to understand this phenomenon, and to predict the behavior of spoilage microflora reducing microbiological stability of final products.

## REFERENCES

[1] Lawlor K, Schuman J, Simpson P, Taormina J. Microbiological spoilage of beverages In: Sperber, W.H., Doyle, M.P. (Eds.) Compendium of the microbiological spoilage of foods and beverages, food microbiology and safety, Springer, New York 2009: 245-83. http://dx.doi.org/10.1007/978-1-4419-0826-1_9

[2] Roethenbaugh G. Trends in beverage markets. In: Ashurst P.R. (Eds.) Chemistry and technology of soft drinks and fruit juices. Oxford, UK: Blackwell Publishing, 2005; 15-34.

[3] Tribst AA, Sant'Ana Ade S, de Massaguer PR. Review: Microbiological quality and safety of fruit juices-past, present and future perspectives. Crit Rev Microbiol, 2009; 35: 310-339. http://dx.doi.org/10.3109/10408410903241428

[4] Juvonen R, Virkajärvi V, Priha O, Laitil A. Microbiological spoilage and safety risks in non-beer beverages produced in a brewery environment. VTT Tiedotteita-Research Notes 2599. 2011.

[5] Kregiel D. Attachment of Asaia lannensis to materials commonly used in beverage industry. Food Control 2013; 32: 537-542. http://dx.doi.org/10.1016/j.foodcont.2013.01.037

[6] Antolak H, Czyzowska, A, Kregiel D. Adhesion of Asaia bogorensis to glass and polystyrene in the presence of cranberry juice. J Food Prot 2015; 78: 1186-1190. http://dx.doi.org/10.4315/0362-028X.JFP-14-440

[7] Kregiel D, Rygala A, Libudzisz Z, Walczak P, Oltuszak-Walczak E. Asaia lannensis - the spoilage acetic acid bacteria isolated from strawberry-flavored bottled water in Poland. Food Control 2012; 26: 147-150. http://dx.doi.org/10.1016/j.foodcont.2012.01.020

[8] Hammes F, Vital M, Egli T. Critical evaluation of the volumetric "bottle effect" on microbial batch growth. Appl Env Microbiol 2010; 76: 1278-81. http://dx.doi.org/10.1128/AEM.01914-09

[9] Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nat Rev Microbiol 2010; 8: 15-25. http://dx.doi.org/10.1038/nrmicro2259

[10] Bischofberger T, Cha SK, Schmitt R, Konig B, Schmidt-Lorenz W. The bacterial flora of non-carbonated, natural mineral water from the springs to reservoir and glass and plastic bottles. Int J Food Microb 1990; 11: 51-71. http://dx.doi.org/10.1016/0168-1605(90)90039-8

[11] Kregiel D, Otlewska A, Antolak A. Attachment of Asaia bogorensis originating in fruit-flavored water to packaging materials. Bio Med Res Int 2014 http://dx.doi.org/10.1155/2014/514190

[12] Horsáková I, Voldřich M, Čeřovský M, Sedláčková P, Šicenrová P. Asaia sp. as a bacterium decaying the packaged still fruit beverages. Czech J Food Sci 2009; 27: 362-365.

[13] Ramos M, Valdez A, Mellinas AC, Garrigos MC. New trends in beverage packaging systems: A review Beverages 2015; 1: 248-272.

[14] Siracusa V. Food packaging permeability behaviour: A report. Int J Polym Sci 2012. http://dx.doi.org/10.1155/2012/302029

[15] Kregiel D. Health safety of soft drinks: contents, containers, and microorganisms. Bio Med Res Int 2015. http://dx.doi.org/10.1155/2015/128697

Received on 30-06-2016 Accepted on 22-07-2016 Published on 28-07-2016

http://dx.doi.org/10.15379/2408-9826.2016.03.02.05