INCIDENCE OF *ALICYCLOBACILLUS* SPP. IN POLISH APPLE AND DARK BERRY JUICE CONCENTRATES AND THE ABILITY OF ISOLATED *A. ACIDOTERRESTRIS* STRAINS TO SPOILAGE OF THESE JUICES

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Summary

Alicyclobacillus spp. is an important thermoacidophilic, spore-forming spoilage bacterium that is a major concern for beverage and juice industries. This study was undertaken to: (i) estimate the incidence of *Alicyclobacillus* spp. and *A. acidoterrestris* in Polish apple and dark berry juice concentrates, and (ii) evaluate the ability of isolated *A. acidoterrestris* strains to spoilage of these juices after dilution to single strength.

Polish apple and berry juice concentrates were screened for the presence of *Alicyclobacillus* spp. between 2002 and 2015. Incidence of *Alicyclobacillus* spp. in apple juice concentrates (n=1164) range from 27.3 to 86.8%, depending on the year. The species *A. acidoterrestris* accounted, depending on the year, from 12.7 to 100.0% of all isolated strains.

Among the dark berry juice concentrates (n=146), approximately 60.0% were contaminated by *Alicyclobacillus* spp. Incidence of *A. acidoterrestris* strains in dark berry juice concentrates range from 4.2% in blackcurrant up to 40.0% in raspberry.

Single strength apple juice promoted the growth of eight of the tested *A. acidoterrestris* strains (> 7 log cfu/ml) when was incubated at 45°C. No *A. acidoterrestris* growth was observed in single strength chokeberry, raspberry, strawberry, cherry and blackcurrant juices during 28 days incubation at 45ºC.

Key words: *Alicyclobacillus acidoterrestris, Alicyclobacillus* spp., apple juice, dark berry juices

INTRODUCTION

Since the early 1980s, when the spoilage of apple juice by a thermoacidophilic, spore forming bacteria [Cerny et al. 1984] was first reported, *Alicyclobacillus* has been recognized as a significant spoilage organism in the fruit juice industry.

Spoilage is characterized by a phenolic off-odour associated with the presence of guaiacol, 2,6-dibromophenol and 2,6-dichlorophenol [Pettipher et al. 1997; Orr et al. 2000; Jensen, Whitfield 2003; Gocmen et al. 2005], and „cheese-like" off-aroma identified as 3-methylbutyric acid and 2-methylbutyric acid [Danyluk et al. 2011].

Due to the number of spoilage episodes and incidences, *Alicyclobacillus acidoterrestris* is recognized as being the most important species and suggested as target of pasteurization in fruit juices and concentrates [Silva et al. 2000]. *A. acidoterrestris* has been isolated from many juices and concentrates including apple, orange, pear, cherry, grapefruit, mango, tomato, white grape, aloe vera, pineapple, lemon, passion fruit, coconut cream, blueberry, pomegranate [Eguchi et al. 2001; Witthuhn et al. 2006; Sokołowska 2009; McKnight et al. 2010; Danyluk et al. 2011] and kiwi [Zhang et al. 2013]. These bacteria were also isolated from various types of acidic beverages [Yamazaki et al. 1996; Sokołowska 2009], including ice tea [Baumgart et al. 1997; Duong and Jensen 2000] and also from beverage ingredients such as sugar [Durak et al. 2010].

However, the presence of these bacteria in juice does not always result in product spoilage. The behaviour (growth, survival or inactivation) of *Alicyclobacillus* species is greatly affected by juice type. Splittstoesser et al. [1994] have investigated the growth of heat-activated spores of two *A. acidoterrestris* strains, in different types of fruit juices. The inoculated strains grew very well in apple, tomato and white grape juices. Very strong growth inhibition was observed in red grape juice, prune juice, cranberry cocktail and mixed fruit juices: apple/grape/cherry, apple/raspberry/grape, apple/red grape. A similar study was carried out by Walls and Chuyate [2000]. The growth of *A. acidoterrestris* was observed in apple, orange, pear, white grape, tomato and grapefruit, but not in pineapple and apple-cranberry juices.

Also Goto [2007], tested the growth behaviour of several strains of *A. acidoterrestris* in a variety of fruit juices, and concluded that the behaviour of the strains depended on the type of juice and also on the source of isolation of the strains. A recent study showed the ability of *A. acidoterrestris* to grow in mango and pineapple juice [Danyluk et al. 2011].

Poland is an important world producer of apple and dark berry juice concentrates. Knowledge of the incidence of *Alicyclobacillus* in these juices and their ability to survive and grow in these products is of major importance in designing the manufacturing process and control measures.

This study was undertaken to: (i) estimate the incidence of *Alicyclobacillus* spp. and *A. acidoterrestris* in Polish apple and dark berry juice concentrates, and (ii) evaluate the ability of isolated *A. acidoterrestris* strains to spoilage of these juices after dilution to single strength.

MATERIALS AND METHODS

Detection of *Alicyclobacillus* spp. in juice concentrates

The International Federation of Fruit Juice Producers' IFU No. 12 (2007) method was used to detect *Alicyclobacillus* strains in juice concentrates. The membrane filtration (Millipore Corp. 0.45 μm) method and enrichment method were applied. Between 2002 and 2015, one thousand hundred sixty four samples of Polish apple juice concentrates (67-73°Bx) and hundred forty six samples of dark berry juice concentrates, including forty eight samples of cherry (64-68°Bx), twenty four of strawberry (63-68 °Bx), eight of raspberry (64-65°Bx), thirty eight of blackcurrant (65-67°Bx) and twenty eight of chokeberry (65-66°Bx) juice, were screened for the presence of *Alicyclobacillus* spp. Presumptive *Alicyclobacillus* were selected for further examination. Confirmation of *A. acidoterrestris* was based on the use of erythritol, with acid production and guaiacol production [Sokołowska et al. 2010].

A. acidoterrestris growth in single strength juices

Tested strains

Eight strains of *A. acidoterrestris* previously isolated in our laboratory were used: 21/11, 175/11 (from cherry juice concentrates); 216/11 (from raspberry juice concentrate); 74/08 (from blackcurrant juice concentrate); TO-57/1/04 (from beverage emulsion) and TO-29/4/02, TO-117/02, TO-169/06 (from apple juice concentrates).

These *A. acidoterrestris* strains used in this study can grow in a temperature range from 20 to 55°C with an optimum between 37 and 50°C and a pH from 2.5 to 6.0 with the optimum between 3.5 and 5.0 [Sokołowska et al. 2010]. A spore suspension of each strain was prepared as described by Sokołowska et al. [2012]. The number of spores was evaluated after serial dilutions and subsequent incubation on Bacillus acidoterrestris-agar (BAT-agar) (Merck) for 5 days at 45 °C. The spread plate method in duplicate was used.

Juice sample preparation and storage

Juice concentrates were diluted with sterile deionized water to single strength juices according to the Association of the Industries of Juices and Nectars from Fruits and Vegetables of the European Union, AIJN, Code of Practice [2012], (chokeberry to 15.0ºBx, pH 3.56; cherry to 13.5ºBx, pH 3.51; blackcurrant to 11.6 ºBx, pH 2.85; apple to 11.2ºBx, pH 4.2 and raspberry and strawberry to 7.0ºBx, pH 3.17 and pH 3.64, respectively). All single strength juices were inoculated with *A. acidoterrestris* spores to 5 log cfu/ml (each strain separately) and then were submitted to a thermal treatment at 80 ºC±1 ºC for 10 min (heat-activation of germination), using a thermostatic water bath. Then, plastic containers a' 125 ml were filled with 20 ml of inoculated juices and incubated at 45°C (according to IFU No 12) for 28 days. Control samples not inoculated were also incubated at the same conditions. Enumeration was carried out at 0, 2, 7, 14, 21 and 28 days, using 3 replicates of juice samples for each storage time, each kind of juice and for each strain. The spread plate method in duplicate on BAT-agar with incubation at 45 °C for 5 days, was used.

Data analysis

An analysis of the variance and Duncan's multiple-range test, using StatSoft® Statistica 7.1, was used to test the significance of the differences ($p<0.05$) between log numbers of microorganisms in the juices along storage time. The means plus minus standard deviations were plotted for each strain/juices and storage time.

RESULTS AND DISCUSSION

Incidence of *Alicyclobacillus* spp. in different fruit juice concentrates

The number and types of juice concentrates analyzed between 2002 and 2015 for the presence of *Alicyclobacillus* spp. and *A. acidoterrestris* are presented in Tables 1 and 2.

Table 1. Incidence of *Alicyclobacillus* spp. and *A. acidoterrestris* in Polish apple juice concentrates produced between 2002 and 2015

Year	Number of tested samples	Percentage of positive samples for *Alicyclobacillus* spp. [%]	Percentage of *A. acidoterrestris* in positive samples [%]
2002	7	71.4	100.0
2003	14	35.7	100.0
2004	22	27.3	50.0
2005	61	50.8	50.0
2006	197	86.8	55.6
2007	99	76.8	16.0
2008	129	74.4	12.7
2009	113	63.7	33.8
2010	84	71.4	33.9
2011	104	69.2	13.9
2012	165	49.1	17.3
2013	82	63.4	13.5
2014	47	70.2	24.2
2015	40	67.5	59.2
Total	1164	-	-

Most of the apple juice concentrates samples, in the range from 27.3 to 86.8%, depending on the year, were positive for *Alicyclobacillus* spp., which are a high numbers, taking into consideration, for example, that Oteiza et al. [2011], only found 11.4 % of apple juice concentrates and pulps (n=5287) produced in Argentina positive for this bacteria. The samples used as the material for our study were delivered by a limited number of manufactures, mainly for control purpose when problems with contamination were suspected, so the results are not representative of the quality of the juices produced across Poland. The high incidence of *Alicyclobacillus* spp. in apple juice concentrates (Table 1) may also be related to the direct contact of these fruit with soil during the harvesting period, since windfall fruits are also used in juice production. The role of soil as a source of *Alicyclobacillus* spp. spores has been well described in the literature [Goto et al. 2008; Groenewald et al. 2009; Wang et al. 2010].

The species *A. acidoterrestris*, which can cause the spoilage of pasteurized fruit juices and beverages, accounted, depending on the year from 12.7 to 100.0% of all the strains isolated from apple juice concentrates. Research conducted by Durak et al. [2010] showed that *A. acidoterrestris* was more frequently associated with apple juice and concentrate than other *Alicylobacillus* spp. The authors found that 66.7% of total apple isolates (n=36) were

A. acidoterrestris strains. So in our study the average percentage of *A. acidoterrestris* strains was lower - 31.3%, but it should be taken into account that number of samples in our research was higher.

Table 2. Incidence of *Alicyclobacillus* spp. and *A. acidoterrestris* in Polish dark berry juice concentrates between 2005 and 2015

Type of juice concentrates	Number of tested samples	Percentage of positive samples for *Alicyclobacillus* spp. [%]	Percentage of *A. acidoterrestris* in positive samples [%]
Cherry	48	60.4	13.8
Strawberry	24	62.5	6.7
Raspberry	8	62.5	40.0
Blackcurrant	38	63.1	4.2
Chokeberry	28	57.1	25.0
Total	146	-	-

The samples of dark berry juice concentrates analysed between 2005 and 2015 (Table 2) were heavily contaminated by *Alicyclobacillus* spp. The percentage of positive samples for these juices ranged from 57.1 (chokeberry juice concentrates) to 63.1% (blackcurrant juice concentrates). Oteiza et al. [2011] found that 83.3 % of strawberry juice concentrates (n=6) produced in Argentina was positive for *Alicyclobacillus* spp. Similarly, in the present study strawberry juice concentrates were also highly contaminated. The high incidence of *Alicyclobacillus* spp. in this kind of juice may be related to the direct contact of these fruit with soil during the cultivation period.

Incidence of *A. acidoterrestris* strains in dark berry juice concentrates range from 4.2% in blackcurrant, and up to 40.0% in raspberry, relative to all of the strains isolated from these juices.

No data could be found in the literature describing the presence of *Alicylobacillus* spp. and *A. acidoterrestris* in raspberry, blackcurrant and chokeberry juice concentrates, in the production of which Poland has a significant world market share. Therefore, this might be the first study reporting the contamination of these juices with *Alicyclobacillus* spp. and the presence of spoilage species of *A. acidoterrestris*.

Growth of *Alicyclobacillus acidoterrestris* in single strength juices

A. acidoterrestris does not grow in juice concentrates and survives mainly as spores [Walls, Chuyate 2000; Sinigaglia et al. 2003; Sokołowska, Łaniewska-Trockenheim 2008] but when a concentrate is diluted to produce single strength juice, the spores may find in such product a favourable environment for germination and growth that, under certain conditions, can lead to product deterioration. So, the growth studies were only carried out in the single strength juices.

The changes in *A. acidoterrestris* populations over a period of 28 days of incubation at 45ºC in apple, chokeberry, raspberry, strawberry, cherry and blackcurrant single strength juices are shown in Figs 1 - 6 respectively. The growth of *A. acidoterrestris* was observed only in apple juice (Fig. 1). After two days of incubation at 45 ºC, the population of all strains achieved levels of > 7 log cfu/ml. The growth curves of all strains are similar to the growth described in orange juice [Gocmen et al. 2005; Goto 2007], mango and pineapple juices [Danyluk et al. 2011], and kiwi juice [Zhang et al. 2013]. Uninoculated control samples had an undetectable *Alicyclobacillus* population (<10 cfu/ml) over the incubation period (data not shown). An unusual „mature cheese-like" odour appeared in apple juice during the bacterial growth or several days later [Sokołowska et al., 2013]. There was no guaiacol odour. The compounds responsible for the „cheese-like" off-aroma were identified by Danyluk et al. [2011] as 3-methylbutyric acid and 2-methylbutyric acid. It can therefore be concluded that *A. acidoterrestris* might contain enzyme systems, possibility related to anaerobic respiration, capable of producing volatiles other than guaiacol and halogenated compounds, provided the substrate is available.

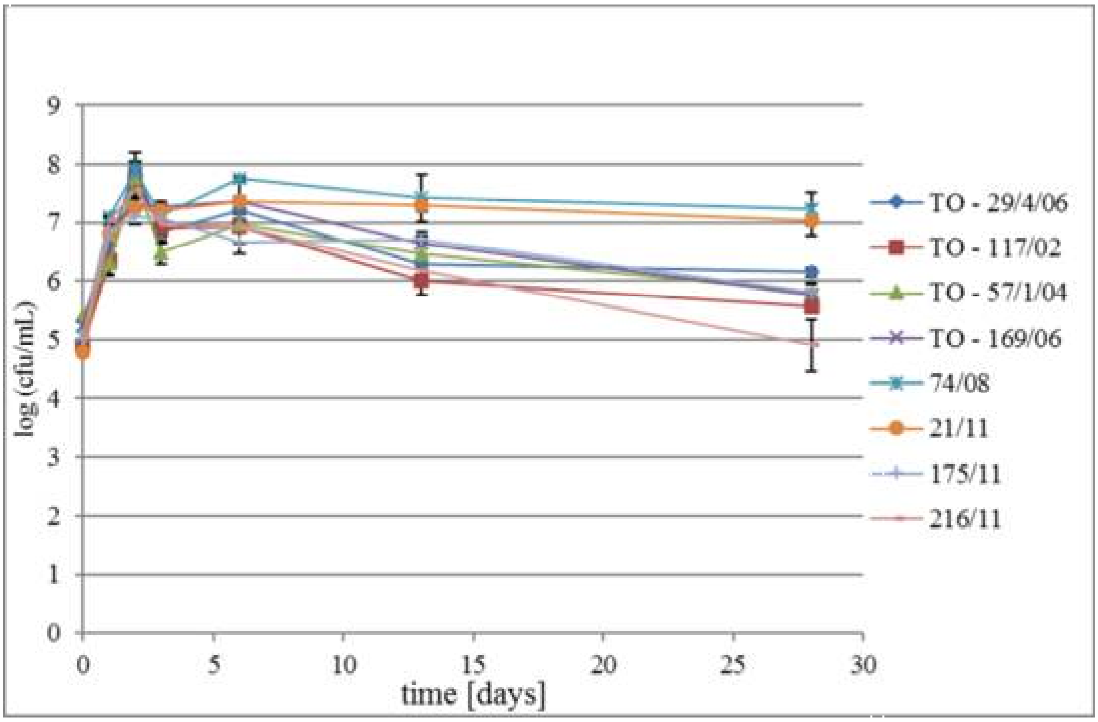

Figure 1. Changes in the populations of eight *A. acidoterrestris* strains in apple juice (11.2°Bx, pH 4.2) during the incubation at 45°C

No *A. acidoterrestris* growth was observed in dark berry juices during 28 days incubation at 45ºC. The survival curves obtained in chokeberry and raspberry juices (Figs. 2, 3) are similar. The population of *A. acidoterrestris* in these juices was reduced by 1.67 to 3.63 log and by 1.45 to 3.15 log, respectively, after 28 days, depending on the strain. At the end of incubation time, in strawberry juice reduction achieved 1.04 to 4.34 log, depending on the strain (Fig. 4). Significant differences in the population of individual strains were found in this juice. The reductions were significantly higher ($p<0.05$), in all data points, for two strains: TO-29/4/02 and TO-57/1/04. However, the behaviour of the strains did not depend on the source of isolation of the strains. Similar reduction was observed [Silva et al. 2000] in cupuaçu pulp during 1 month storage in aerobic and anaerobic conditions.

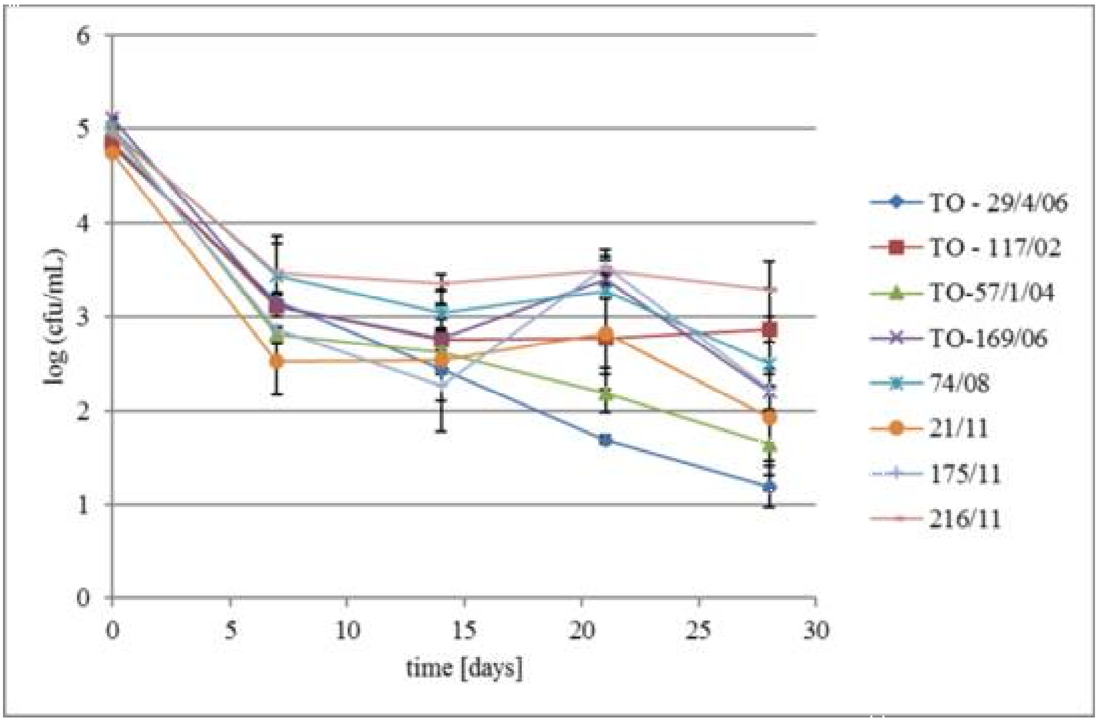

Figure 2. Changes in the populations of eight *A. acidoterrestris* strains in chokeberry juice (15.0 °Bx, pH 3.56) during the incubation at 45°C

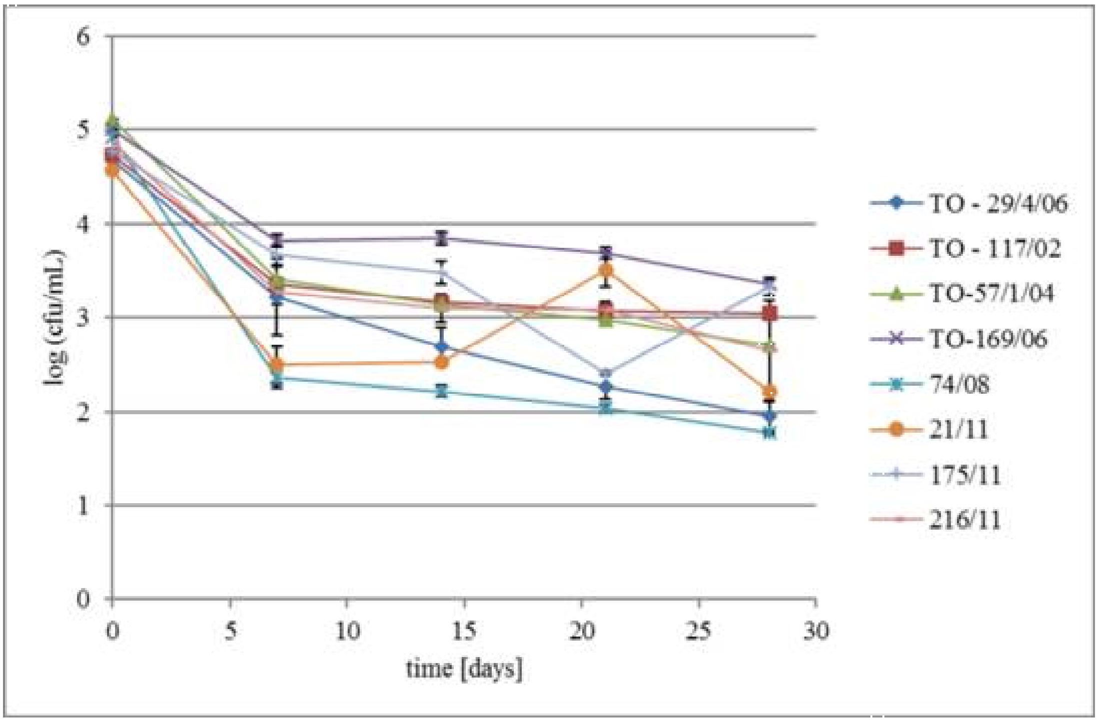

Figure 3. Changes in the populations of eight *A. acidoterrestris* strains in raspberry juice (7.0 °Bx, pH 3.17) during the incubation at 45°C

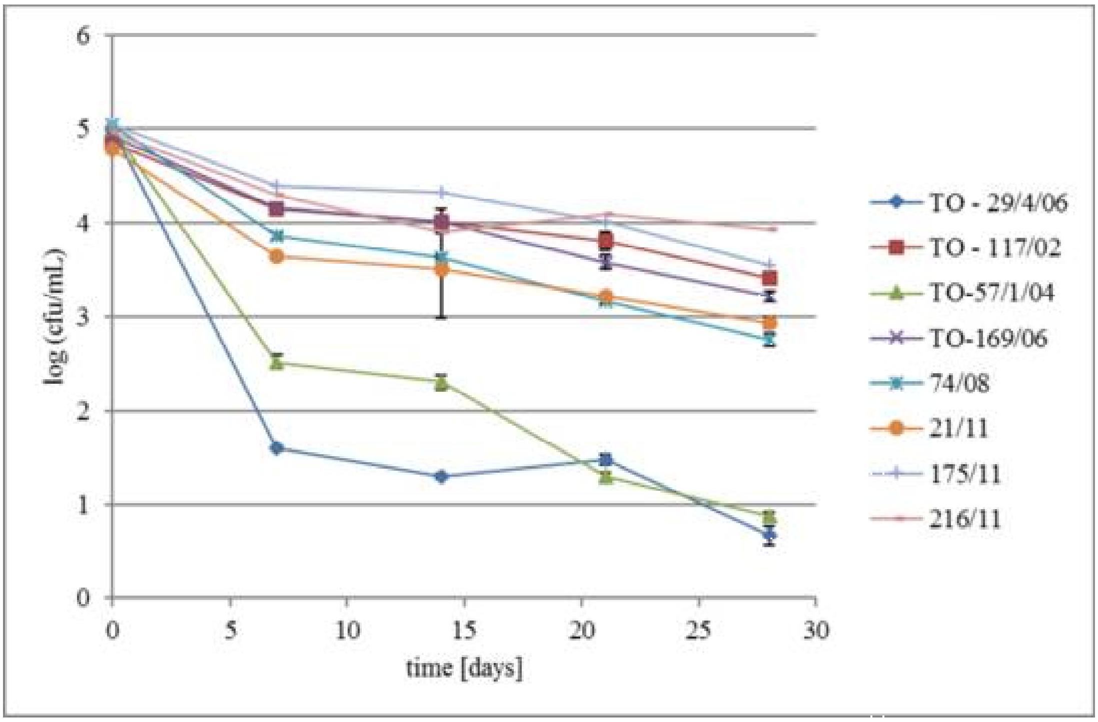

Figure 4. Changes in the populations of eight *A. acidoterrestris* strains in strawberry juice (7.0 °Bx, pH 3.64) during the incubation at 45°C

In cherry and blackcurrant juices, the population of *A. acidoterrestris* decreased systematically during 28 days of incubation, reaching reduction even up to 5.0 log (Figs. 5, 6). Significant differences in the survival population of individual strains in these juices, after 21 and 28 days of incubation, were observed. In cherry juice (Fig. 5) after 21 days of incubation reduction was significantly lower ($p<0.05$) for TO-169/06 and 216/11 strains, and reached 1.54 and 1.81 log, respectively. After 28 days of incubation, only for one strain: 216/11, the significantly lower ($p<0.05$) reduction was observed, and it was 2.30 log. The reduction were significantly lower ($p<0.05$), and reached 2.5 – 3.2 log, for TO-117/02, TO-169/06 and 216/11 strains, in blackcurrant juice after 28 days of incubation (Fig. 6). The behaviour of the strains did not depend on the source of isolation of the strains.

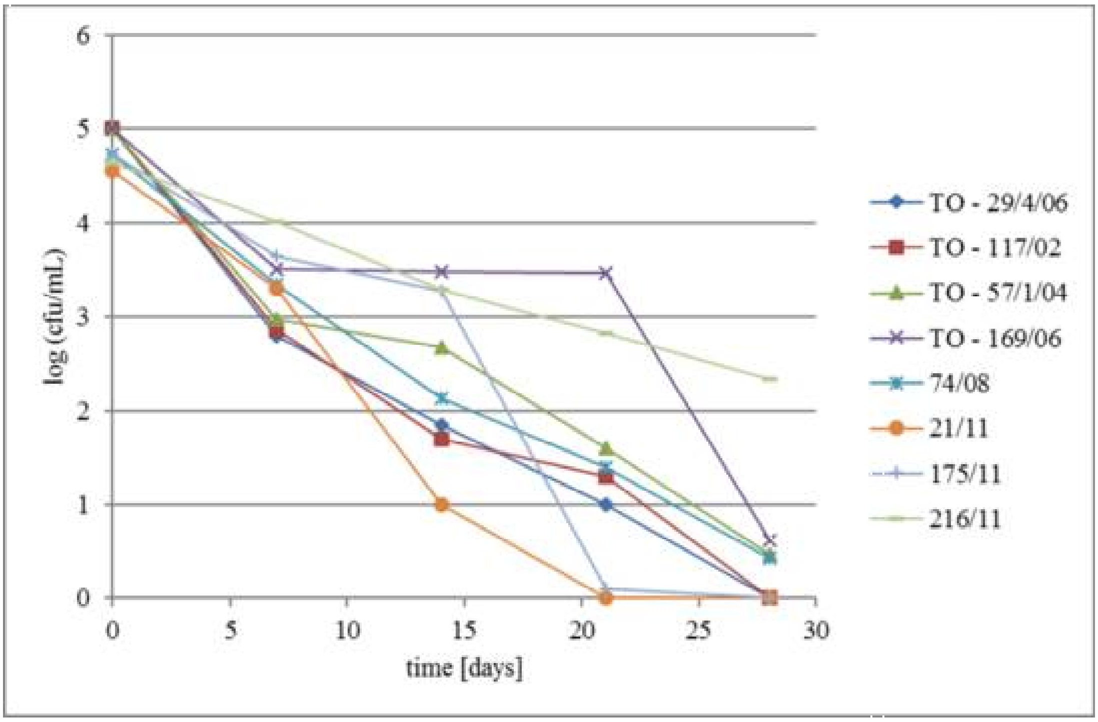

Figure 5. Changes in the populations of eight *A. acidoterrestris* strains in cherry juice (13.5 °Bx, pH 3.51) during the incubation at 45°C

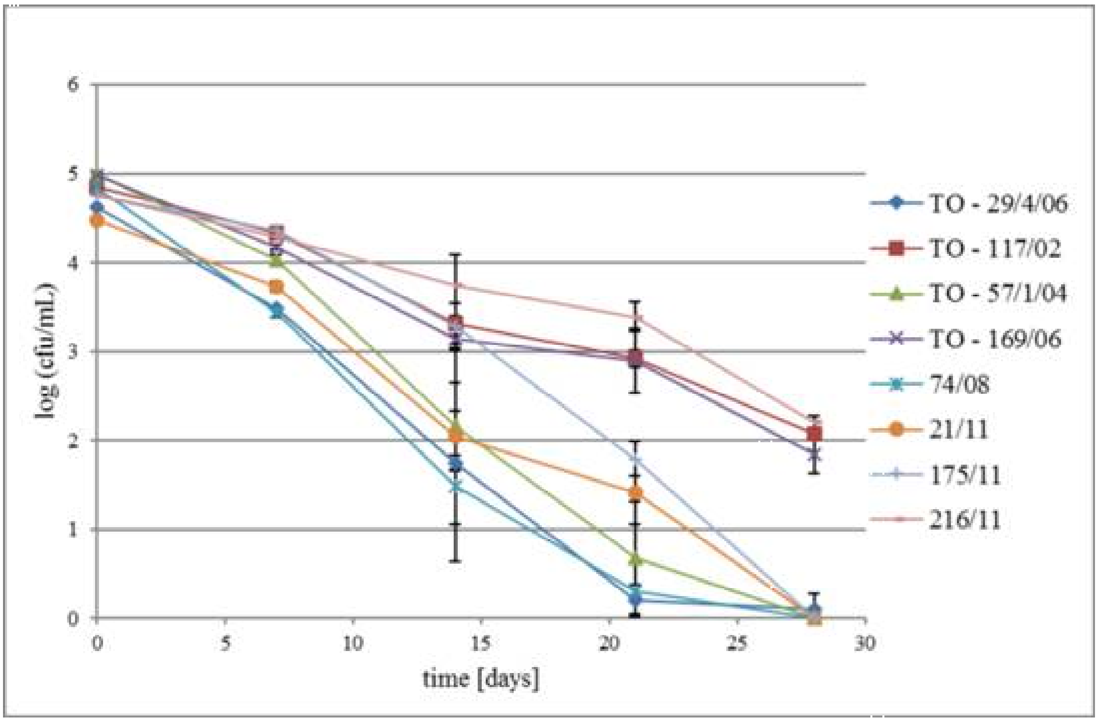

Figure 6. Changes in the populations of eight *A. acidoterrestris* strains in blackcurrant juice (11.6°Bx, pH 2.85) during the incubation at 45°C

Berry fruits are rich sources of bioactive compounds, such as phenolics and organic acids, which have antimicrobial activities against many microorganisms, including human pathogens. The antimicrobial activities of blueberry and blackberry against foodborne pathogens, including *Listeria monocytogenes*, *Salmonella* Typhimurium, *Campylobacter jejuni* and *Escherichia coli* O157:H7 [Biswas et al. 2012; Yang et al. 2014] were demonstrated. The antimicrobial activity of eight Nordic berries (bilberry, lingonberry, cranberry, red raspberry, strawberry, cloudberry, blackcurrant and sea-buckthorn berry) and berry phenolics were reported by Puupponen-Pimiä et al. [2005a]. In this report, it was found that phenolic compounds, especially ellagitannins of berries, were strong inhibitory compounds against foodborne pathogens (*Staphylococcus aureus*, *Salmonella* Typhimurium). Several mechanisms in the growth inhibition of bacteria were involved, such as destabilisation of the cytoplasmic membrane, permeabilisation of the plasma membrane, inhibition of extracellular microbial enzymes, direct actions on microbial metabolism and deprivation of the substrates required for microbial growth [Puupponen-Pimiä et al. 2005b].

There are no data in the literature regarding the mechanism of the antimicrobial activities of these compounds against spores, however inhibition of spore germination or inhibition of the outgrowth of germinated spores is possible.

CONCLUSIONS

The data presented in this study contributes to the ecology of *Alicyclobacillus* spp. found in fruit juices produced in Poland. The isolation of *A. acidoterrestris* from juice concentrates reinforces the relevance of minimizing spoilage risks by preventing contamination with *A. acidoterrestris* by applying good agricultural practices, mainly in the fruit inspection, selection and washing steps, as well as by applying adequate control measures during fruit juice production. Although berry juices may contain *A. acidoterrestris* spores, they do not undergo spoilage, so added to others juices may prevent growth of these bacteria, but on the other hand they can be a source of contamination.

Knowledge of the prevalence of *Alicyclobacillus* strains in juice concentrates and their behaviour after dilution is of major importance in designing the manufacturing process and control measures.

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