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IDENTIFICATION AND STUDY OF THE BEHAVIOR OF *S. AUREUS* AND *S. EPIDERMIDIS* IN FRESH AND FROZEN STRAWBERRIES

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ABSTRACT

Background. Methicillin-Resistant *Staphylococcus aureus* (MRSA), which is resistant to many antibiotics, creates a serious problem for human health if present in food. This study aimed to assess the quality of commercially-available fresh and frozen strawberries and to compare the behavior of staphylococci in these fruits as affected by the temperature of freezing.

Material and methods. The research material included different species of fresh strawberries and strawberries frozen with the fluidization method to –40°C and packaged in an industrial environment. These were checked for the presence of *Staphylococcus aureus* resistant to methicillin (MRSA). The strawberries were purchased at food markets in the Tricity and were divided into two groups (A and B). Group A was analyzed without washing, group B was washed in sterile, distilled water for 15 minutes. The strawberries were placed in sterile PE/PA bags. Then 1 mL of *Staphylococcus aureus* Rosenbach ATCC 25923 and *Staphylococcus epidermidis* ATCC 12228 of known inoculum were added to each bag (except the control samples). The samples were mixed thoroughly and then hermetically sealed. The samples were then frozen and stored in the freezer at a temperature of –18 ±2°C for 2 months. In the material being tested *Staphylococcus aureus* was cultured in selective Baird-Parker RPF Agar. Incubation was carried out at a temperature of 37°C for 48 hours in sterile conditions. After 48 hours of incubation, characteristic colonies were transferred onto the reaction field of the PROLEX™ STAPH XTRA LATEX KIT.

Results. The results obtained show that 1/3 of the samples of commercial strawberries analyzed were colonized by methicillin-resistant *Staphylococcus aureus* (MRSA). The process of fruit washing was observed to reduce the number of samples containing MRSA from 11.7 to 8.3%. There was no significant difference in the size of the *S. aureus* ATCC 25923 population after freezing the strawberries at –18°C, depending on the particular washing process for these fruits. The analysis of strawberries frozen with the fluidization method at –40°C showed a minimum contamination degree with *S. aureus* after the period of storage.

Conclusions. Studies have shown that MRSA are present in 15.4% of strawberries obtained from the field. Storing strawberries frozen at –18ºC causes a reduction in the number of *S. aureus* by 0.16 $\log_{10}$CFU/g and *S. epidermidis* by 0.47 $\log_{10}$CFU/g when they were subjected to rinsing after harvesting. Effective inhibition of MRSA in strawberries is obtained when fluidization technology is applied at –40ºC.

Keywords: strawberry, frozen state, staphylococci, MRSA

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INTRODUCTION

Nutritionists recommend the consumption of a considerable amount of fresh fruit as a source of vitamins and natural substances necessary for the body to function properly. Due to antioxidants and antibacterial substances present in strawberries, these are considered to be fruits with health-promoting properties (Akyiama et al., 2001; Bojarska et al., 2006; Cordenunsi et al., 2005; Lee et al., 2003; Rochalska et al., 2011). The way of harvesting berries is a crucial operation affecting their health safety. The risk posed by microflora present in food products may result from the quality of water, field sanitation, workers health and hygiene, as well as packaging sanitation (Barth et al., 2009).

The microflora isolated during strawberry testing included: moulds, *E. coli*, *Bacillus cereus*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* (Sivapalasingam et al., 2004; Siro et al., 2006; Yoon et al., 2010).

Literature data confirms the likely presence of microorganisms such as *Salmonella*, *Listeria monocytogenes*, and *Bacillus cereus* on the surface of strawberries (Delbeke et al., 2013; Harris et al., 2003; Johannessen et al., 2002; Knudsen et al., 2001). In the case of packaged strawberries *Geotrichum*, *Saccharomyces*, *Torulopisis*, *Candida*, *Hansenula*, *Pseudomonas, Bacillus* and also lactic acid bacteria were isolated. MAP-packed strawberries, on the other hand, were found to be colonized by yeasts from the genera: *Cryptococcus* and *Rhodotorula* (Barth et al., 2009; Jay et al., 2005).

The aim of this study was to assess the quality of commercially-available fresh and frozen strawberries and to compare the increase or reduction in staphylococci in these fruits as affected by the freezing temperature.

MATERIALS AND METHODS

The research was carried out in three stages

I. Determination of the staphylococci population in strawberries available on the market
II. Determination of the counts of *S. aureus*, MRSA and *S. epidermidis* in strawberries that were stored frozen at a temperature –18 ±2°C
III. Determination of the counts of staphylococci (*S. aureus*, MRSA and *S. epidermidis*) in strawberries frozen with the fluidization method to –40°C and packaged in an industrial environment.

Inoculation, treatment, product preparation, and storage

Part of the research material were strawberries frozen with the fluidization method to –40°C and packaged in an industrial environment, which were checked for the presence of *Staphylococcus aureus* resistant to methicillin (MRSA). These strawberries originated from 7 different producers ($n = 32$). In this material, *Staphylococcus aureus* was cultured in selective Baird-Parker RPF Agar (BioMerieux, France). Incubation was carried out at a temperature of 37°C for 48 hours in sterile conditions. After 48 hours of incubation, characteristic colonies (color – black, fibro halo – presence) of (1–4) *Staphylococcus aureus* ATCC 25923 were transferred using a sterile loop onto the reaction field of the PROLEX™ STAPH XTRA LATEX KIT (BIOCORP) and analyzed according to the kit producer's instructions.

Microbiological analyses were carried out with the culture method on Baird-Parker RPF after prior dilution of the samples in accordance with the relevant methodological standards.

The research material included fresh strawberries (*Fragaria ananassa*) purchased at food markets in the Tricity (Poland). The fruits originated from: southern ($n = 20$) and northern ($n = 36$) Poland. Two types of strawberries were studied: greenhouse ($n = 14$) and field ($n = 42$). Fresh Kashubian strawberries were analyzed for the presence of *Staphylococcus epidermidis* and *Staphylococcus aureus* resistant to methicillin MRSA. Part of the samples were washed in sterile, distilled water for 15 minutes in order to clean the fruit surfaces of impurities, and the remaining part of the samples was analyzed without the washing step.

Twenty grams of both washed and unwashed strawberries were left for testing before the process of freezing, and the remaining part was divided according to the scheme (Fig. 1).

After placing 100 g of strawberries in sterile PE/PA bags designed for food freezing, 1 mL of *Staphylococcus aureus* Rosenbach ATCC 25923 (collection Merck) and *Staphylococcus epidermidis* (collection Biocorp) ATCC 12228 of known inoculum were added to each bag (except the control samples). The samples were mixed thoroughly and then

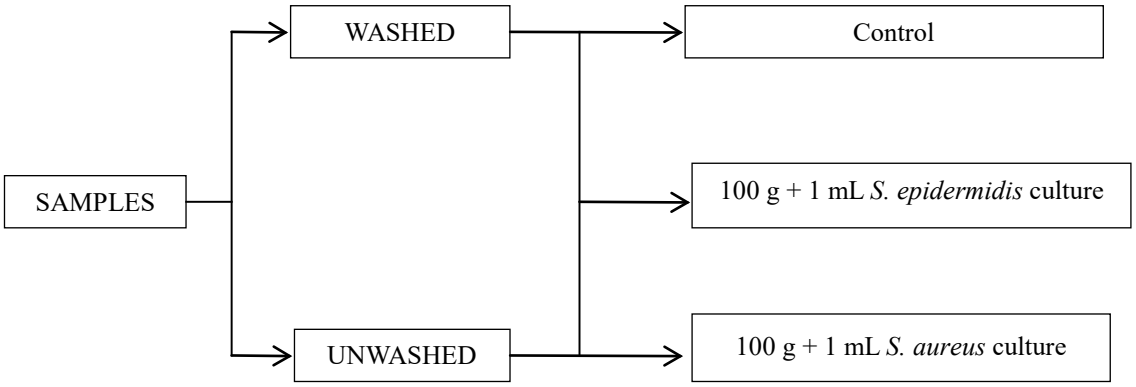

Fig. 1. Scheme describing inoculation of strawberry samples

hermetically sealed by using a "Severin" vacuum packager. These samples were frozen and stored in the freezer at a temperature of –18 ±2°C for 2 months (60 days). The counts of *Staphylococcus aureus* and *Staphylococcus epidermidis* were determined on selective Baird-Parker RPF Agar (BioMerieux, France). Incubation was carried out at a temperature of 37°C for 48 hours. Afterwards, characteristic colonies (color – black, fibro halo – presence) of *Staphylococcus aureus* were transferred using a sterile loop onto the reaction field of the PROLEX™ STAPH XTRA LATEX KIT (BIOCORP) and analyzed according to the kit producer's instructions.

Statistical analysis

The results were subjected to statistical analysis using Statistica v. 10 (StatSoft) software. Student's t-test was applied to analyze the data and to assess the differences in the count of staphylococci during storage depending on the inoculum of bacteria and the process of fruit washing. Linear correlation equations were determined.

Equations describing the change in the number of staphylococci include the following parameters: Y – count of *S. aureus* ATCC 25923 and count of *S. epidermidis* ATCC 12228, x – time of storage, a, b – parameters of the equation.

RESULTS

Analysis of commercial strawberries' revealed that over 38% of fruit samples were characterized by the presence of *S. aureus* (Table 1). The fruit washing step had no significant effect on the reduction in the number of samples with the confirmed presence of these microorganisms (Table 1).

11.8% of the samples of strawberries examined revealed the presence of *S. aureus* MRSA (Tables 1 and 2). However, in all strawberries it was observed that the process of washing reduced the number of MRSA-containing samples from 11.8 to 8.3%. In addition, the study concluded that the level of MRSA in strawberries was dependent on the cultivation system. Strawberries grown in greenhouse conditions were free of MRSA *S. aureus* (Tables 1 and 2). The region in

Table 1. Occurrence of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) in samples of strawberries tested, %

Type of samples	*Staphylococcus aureus*		*Staphylococcus aureus* (MRSA)	
	present	absent	present	absent
Fresh strawberries	38.2	61.8	11.8	88.2
Fresh, washed strawberries	33.3	66.7	8.3	91.7

Table 2. Occurrence of *S. aureus* and methicillin-resistant *S. aureus* (MRSA) in strawberries depending on their type and growing region, %

Type of samples	*Staphylococcus aureus*		*Staphylococcus aureus* (MRSA)	
	present	absent	present	absent
Greenhouse strawberries	28.6	71.4	0	0
Field strawberries	42.3	57. 7	15.4	84.6
Strawberries from southern region	33.3	66.7	8.3	91.7
Strawberries from northern region	27.8	71.2	11.1	88.9

which the strawberries were grown had no significant impact on the presence of antibiotic-resistant staphylococci. The percentage of MRSA-containing samples harvested in southern regions was slightly lower than that of the samples originating from northern regions (Tables 1 and 2).

In order to assess the increase or reduction in the population of staphylococci during storage of the strawberries in the frozen state, the strains of *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 were added to fresh fruits that were then packaged under vacuum conditions. After freezing the packaged fruits to –18°C, staphylococci population was assessed in these products after 60 days of storage in a frozen state.

The average number of *S. aureus* in unwashed strawberries after 2 months of storage amounted to 4.49 $\log_{10}$CFU/g. Washing the fruits caused the average count of *S. aureus* population to reach 4.33 $\log_{10}$CFU/g (Table 3).

The washing step produced no statistically ($p < 0.05$) significant differences in the size of the *S. aureus* ATCC 25923 population after the process of freezing the strawberries. The difference in the size of the population observed between both parts of the fruits (A, B) after 60 days of storage was 0.16 $\log_{10}$CFU/g at $p = 0.691$. The reduction in the number of *S. aureus* ATTC 25923 in frozen unwashed strawberries varied from 2.23 $\log_{10}$CFU/g to 2.99 $\log_{10}$CFU/g.

Differences in the *S. aureus* ATCC 25923 cell count added to strawberries and then isolated after 60 days of storage could be described by using the linear equation shown below:

$$Y = -1.56x + 7.296, \quad r^2 = 0.186$$

where:

Y – count of *S. aureus* ATCC 25923.

In the case of the washed fruits, the minimum reduction of staphylococci amounted to 2.51 $\log_{10}$CFU/g. After 60 days of storage, the maximum decline in the number of staphylococci by 3.68 $\log_{10}$CFU/g was determined. The correlation between the number of

Table 3. Changes in the number of *Staphylococcus aureus* ATCC 25923 during frozen storage for 60 days

Type of strawberries	Time days	Average value	Number of bacteria $\log_{10}$CFU/g		Standard variation
			max	min	
Not washed fruits	0	7.62	7.62	7.62	0.0
	60	4.49	5.39	3.63	0.678
Washed fruits	0	7.62	7.62	7.62	0.0
	60	4.33	5.11	3.94	0.408

Table 4. Changes in the number of *Staphylococcus epidermidis* ATCC 12228 during frozen storage for 60 days

Type of strawberries	Time days	Average value	Number of bacteria $\log_{10}$CFU/g		Standard variation
			max	min	
Not washed fruit	0	7.43	7.43	7.62	0.0
	60	4.95	5.34	3.63	0.678
Washed fruit	0	7.43	7.43	7.62	0.0
	60	4.48	5.72	3.94	0.408

bacteria and the time of storage is presented by the following equation:

$$Y = -1.645x + 7.326 \text{ for } r^2 = 0.193$$

where:

Y – count of *S. aureus* ATCC 25923.

On the basis of these equations, it could be concluded that the dynamics of changes observed for both types of strawberries within those 60 days of storage were similar. The linear correlation coefficients of the equations indicate a greater reduction in the number of staphylococci in the strawberries washed before the freezing process. It was found that the count of staphylococci in the strawberries after frozen storage was highly affected by the inoculum. The coefficient of determination for the correlation equations allowed it to be stated that the process of washing had no significant effect on the level of staphylococci reduction but only impacted on the dynamics of their extinction.

In the case of applying the *S. epidermidis* ATCC 12228 strain, a reduction was observed in the number of staphylococci cells after 60 days, which reached 2.48 $\log_{10}$CFU/g on average for unwashed strawberries (Table 4).

The washed strawberries were characterized by a decrease in *S. epidermidis* ATCC 12228 count over the same period, i.e. by 2.95 $\log_{10}$CFU/g in relation to the inoculum (Table 4).

Changes in the *S. epidermidis* ATCC 12228 count in strawberries stored for 60 days and not washed before freezing can be expressed by the linear correlation:

$$Y = -1.24x + 6.95, \quad r^2 = 0.154$$

where:

Y – count of *S. epidermidis* ATCC 12228.

In the case of fruits washed before the freezing process, the number of staphylococci after 2 months of storage is well described by the linear correlation equation:

$$Y = -1.465x + 6.946, \quad r^2 = 0.161$$

where:

Y – count of *S. epidermidis* ATCC 12228.

The coefficients of determination were established (r^2 = 0.161) for equations describing changes in the populations of *S. epidermis* and *S. aureus*. The coefficients of determination r^2 assayed for both equations indicate that the changes in the number of staphylococci can only to some extent be explained by storage time.

Tissues of strawberries frozen to –18°C and –40°C exhibited different physicasurface l properties in the first phase after the preservation process (Fig. 2).

Studies on frozen strawberries with the application of the fluidization method up to –40°C showed a minimal degree of contamination with *S. aureus* after the storage period. The number of staphylococci ranged from 1.00 to 1.30 $\log_{10}$CFU/g in 18.8% of the samples tested. In other samples of strawberries, these bacteria were absent. Similar results were obtained for *Staphylococcus epidermidis*. Methicillin-resistant staphylococci (MRSA) were not found in any of the fruit samples tested (Table 5).

Table 5. The presence of staphylococci in strawberries frozen at a temperature of –40°C

Producer	*S. aureus*		MRSA		*S. epidemidis*	
	present %	max number of bacteria $\log_{10}$CFU/g	present %	absent %	max number of bacteria $\log_{10}$CFU/g	present %
I K	50	1.30	0	100	0	0
II H	0	0	0	100	0	0
III O	0	0	0	100	0	0
IV F	0	0	0	100	0	0
V D	50	0	0	100	0	0
VI I	50	1.00	0	100	1.00	90
VII G	0	1.00	0	100	1.00	90
VII T	0	1.30	0	100	1.30	50

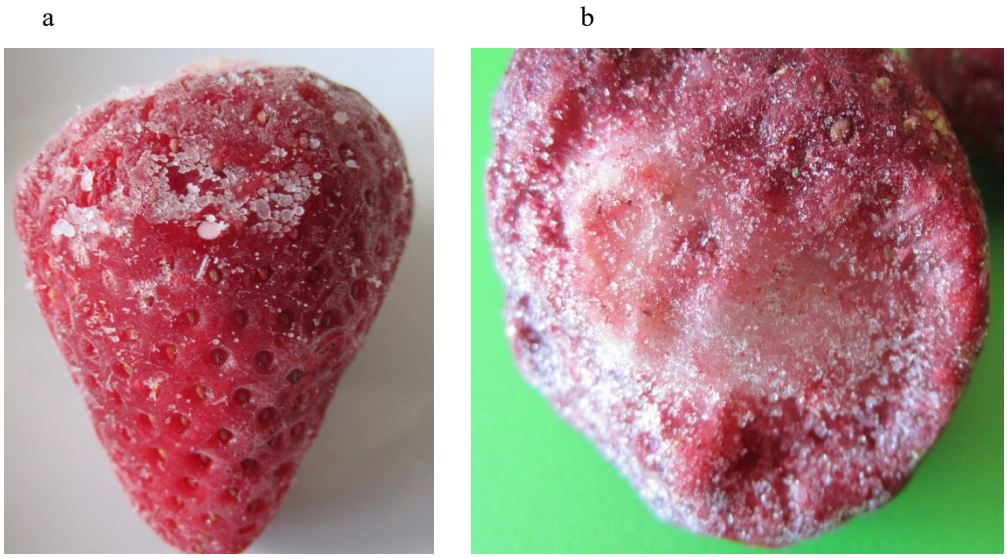

Fig. 2. Fruits frozen: a – at –18°C, b – by the fluidization method at –40°C

DISCUSSION

The most common spoilage of strawberries after harvesting is caused by the presence of the *Botritis cinerea* fungus species. However, the safety of consumers depends on other microbes associated with fruit harvesting and processing. These include, inter alia, *S. aureus*, *Salmonella* or *E. coli* (Delbeke et al., 2013).

Research by other authors have demonstrated that the survival rate of those micro-organisms is dependent on technology of fruit preservation after harvesting, inter alia (Campos et al., 2012; Luksiene and Paskeviciute, 2011; Mali and Grossmann, 2003; Mitcham and Mitchell, 2002; Raybaudi-Massilia et al., 2009).

The strawberries which were the subject of this research were grown in different conditions and varied in terms of the occurrence of MRSA. This may indicate the origin of these bacteria associated with the participation of humans in cultivation, harvesting and growing practices. It is likely that the presence of methicillin-resistant staphylococci in fruits coming from field cultivation only partially resulted from the method of harvesting. The second source of contamination of these fruits could be dust particles, which was indicated by the origin of the isolated strains, e.g. from the soil. Our data showed that the region in which the fruit were grown and harvested did not affect the percentage of samples containing *S. aureus* and MRSA.

Some studies suggest that freezing strawberries affects the level of specific components of these fruits. Changes in the concentration of components of an antibacterial character may be the cause of staphylococci presence after the process of frozen storage (Sahari et al., 2004).

Siro et al. (2006) examined the microflora of frozen strawberries and pointed out that storing these fruits in an atmosphere with a low oxygen concentration or in the MAP system did not result in a reduction in other pathogenic bacteria, such as *E. coli*, *Listeria* and *Salmonella*. However, there needs to be a discussion on whether this is the sole effect of acid activity.

Vacuum packaging of strawberries inoculated with *S. aureus* cells followed by their freezing to –18°C and storage in this state for 60 days resulted in the reduction of *S. aureus* ATCC 23925 count. Staphylococci tolerate the presence of acids and are more resistant to their effects than the bacteria analyzed by Siro et al. (2006).

It should be emphasized that the interaction of low temperatures, a lack of oxygen and storage time at –18°C was the cause of staphylococci population growth inhibition.

Research on the survivability of *L. monocytogenes* in strawberries carried out by other authors has shown that freezing to –20 ±2°C resulted in a decline in the number of these bacteria from 0.5 to 1.8 $\log_{10}$CFU/g after 28 days. At the same time, it was stated that freezing under these conditions allowed these bacteria to survive for 4 weeks (Flessa et al., 2005).

It should also be noted that the extent of *S. aureus* reduction after 60 days observed in this research was significant in comparison to that obtained for other bacteria.

The results obtained in this study for strawberries stored at 18 ±2°C show that washing the fruits before freezing caused no statistically significant difference in the behavior of staphylococci in comparison to their behavior in the unwashed fruits (0.471, $p = 0.264$ for *S. aureus* and 0.161 at $p = 0.691$ adequately for *S. epidermidis*).

The nature of changes in the case of *S. aureus* and *S. epidermidis* presence in the fruits with population numbers exceeding 7 $\log_{10}$CFU/g indicates the existence of a factor other than time that reduced their counts during the 60-day storage period. The reduction in the number of bacteria after 60 days of storage at –18°C varied from 41.1% to 43.2% with regard to inoculum of *S. aureus* and from 33.3% to 39.7% for *S. epidermidis*. The study showed that the reduction in bacteria during frozen storage at –18°C ranged from 33.3% to 43.2% for both strains of staphylococci, but was greater in the case of *S. epidermidis* in the washed strawberries. A greater reduction was observed for *S. aureus* and for strawberries washed before freezing. It should be noted that this could have resulted from minor differences in the structure of the cell wall of *S. aureus* and *S. epidermidis*. There might also have been a slight amount of water on the fruit's surface that remained after washing and contributed to damage to the cell wall of *S. epidermis* in the process of freezing, which eventually caused higher extinction dynamics of cells of that strain. Moreover, surface tension of fruits decreases during the freezing process (Ribeiro et al., 2007).

During the process of freezing, ice crystals are formed on the surface of the fruit tissue (Fig. 2). The dynamics of their formation and, at the same time, their size vary. This is the cause of different surface-tension values noted during the first period of fruit freezing. In addition, a rapid change in water activity on the surface of strawberry tissue during freezing to –40°C causes changes in the osmotic pressure and reduces the staphylococci survival rate.

Literature data shows that *S. aureus* is much more resistant to freezing than *S. epidermidis*, which again may result from the different habitats of both species from which they are usually isolated.

CONCLUSION

Studies have shown that MRSA are present in 15.4% of strawberries obtained from the field. Storing strawberries frozen in the –18ºC causes a reduction in the number of *S. aureus* by 0.16 $\log_{10}$CFU/g and *S. epidermidis* by 0.47 $\log_{10}$CFU/g when they were subjected to rinsing after harvesting. An effective inhibition of MRSA in strawberries is obtained when fluidization technology is applied at –40ºC.

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