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MORPHOSTRUCTURAL DAMAGE OF FOODBORNE BACTERIAL PATHOGENS INFLUENCED BY SWAMP CRANBERRY (*VACCINIUM OXYCOCCOS* L.) POMACE EXTRACT REVEALED BY TRANSMISSION ELECTRON MICROSCOPY (TEM)

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Summary. Foodborne pathogens are a serious threat to human health when they contaminate food products. Nowadays, consumers are interested in natural, safe, multi-health benefits food and at the same time increasingly concerned about food chemical preservatives and additives. Due to this fact, it is important to focus on natural alternatives for food products preservation. Data related to antimicrobial properties of cranberry fruit are available; however, information regarding morphostructural damage due to foodborne pathogens, due to the natural extracts is still scarce. This study was performed to evaluate the morphostructural effects of swamp cranberry water–ethanol pomace extract (WEPE) on selected Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and Gram-negative (*Escherichia coli* and *Salmonella* Enteritidis) foodborne bacterial pathogens. The morphological and ultrastructural alterations of extract-treated bacteria cells were observed using transmission electron microscopy technique (TEM). In our study, TEM revealed changes in the cell structure and the integrity of cells both for Gram-positive and Gram-negative strains. Our investigation revealed substantial structural damage at the cellular level and irreversible cell membrane rupture with the apparent leakage of intracellular contents. Present results indicate that WEPE is highly effective against foodborne pathogens, so as the antimicrobial effect of WEPE, including destructive influence on bacterial cells proved in this study, suggests promising potential use of cranberry extract as a natural preservative in food products. Influence of WEPE on other processes, such as inhibition of nucleic acid synthesis, inhibition of cell wall and membrane protein synthesis or disturbance of cellular energy metabolism, should be the subject of further research.

Key words: cranberry pomace, antibacterial activity, TEM, cell morphostructural changes, bacterial pathogens

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INTRODUCTION

Foodborne pathogens are a serious threat to human health when they contaminate food products. Nowadays, consumers are interested in organic healthy food and do not object using of plant extracts as natural inhibitors on unwanted microbiota in food products. It indicates usefulness of plant extracts as natural food preservatives [Wu et al. 2009]. Many naturally occurring compounds found in plants, including cranberry, exhibited strong antimicrobial activity and serve as a source of antimicrobial agents against many pathogens and highlight their hopeful use as an alternative to chemicals for inhibiting the growth of pathogenic bacteria in food products [Gould 1996, Wu et al. 2008, Côté et al. 2011, Kaviya Srinidhi 2014, Gniewosz and Stobnicka 2017, Stobnicka and Gniewosz 2017]. However, information regarding to morphostructural damage affecting foodborne pathogens, due to the natural extracts is still scarce. Fruit pomace, which is post-production waste from the fruit processing industry, can be used to obtain bioactive compounds that can be used as natural food additives due to their antioxidant, antimicrobial, coloring, or flavoring aspects.

Transmission electron microscopy (TEM) is frequently used method to visualize the ultrastructural damage on cell wall. Ultrathin sections obtained by conventional procedures, namely fixation with aldehydes, post-fixation with osmium tetraoxide, dehydratation and embedding in epoxy resin, allow the observation of membrane and cytoplasmatic alterations, both external and internal changes such as membrane dis-integrity, rupture or detachment, cytoplasmic release and leakage of intracellular contents [Hartmann et al. 2010, Kim and Chung 2011]. TEM technique is widely used for confirmation of antimicrobial properties of natural compounds coming from herbs and plants against bacteria and fungi [Wu et al. 2008, Tyagi and Malik 2010, Yinfeng Lyu et al. 2016].

The aim of this study was to evaluate the morphostructural changes affecting bacterial cells following WEPE treatment.

MATERIAL AND METHODS

Plant material and extract preparation

Cranberry fruit came from the forest areas in the Mazovian Province in Poland (52°4′N; 20°54′E) and were collected during harvesting season (October–November). Extract was prepared according to Stobnicka and Gniewosz [2017].

Bacteria

All strains, Gram-positive: *Staphylococcus aureus* A-529, *Listeria monocytogenes* 17/11 and Gram-negative: *Escherichia coli* O26, *Salmonella* Enteritidis 322/11 were clinical isolates and were received from National Institute of Public Health – National Institute of Hygiene (NIPH-NIH) (Warsaw, Poland).

Preparation of cells for TEM analysis

Bacterial strains were grown in a brain-heart infusion broth (BHI; Merck, Germany) at 37 ±1°C for 18 h. The biomass was centrifuged at 3,750 × g in MiniSpin (Eppendorf, USA) for 10 min at 4°C. Supernatant was discarded and the pellet was washed with 0.85% sterile saline solution. Cellular sediments were washed two times using 0.85% solution of sterile physiological saline. Pellets were suspended in 1 ml saline without and with solution of we-PSCE corresponding with MICs with pH adjusted to 5.2 with 1N NaOH to avoid direct influence of organic acids [Li et al. 2008, Erdem et al. 2016]. Samples were left for 20 min at room temperature (21°C) and then fixed in 2.5% glutaraldehyde (Scharlau, Germany) for 2 h at 4°C, then poured with phosphate buffer (PBS) (Scharlau, Germany) pH 7.2 for 2 h in 4°C. The final fixation was carried out in 1% OsO_4 (Merck, Germany) at 4°C for 1 h. After dehydration with increasing gradient of ethyl alcohol (POCH, Poland) and acetone saturation (POCH, Poland), the samples were sunk at Epon 812 (Spi-Chem, United States). After polymerizing Epon, the samples were cut with a diamond knife on ultramicrotome (LKB, Sweden). Ultrathin sections (70 nm) were transferred to copper nets, which were then contrasted in uranyl acetate (Scharlau, Germany) and lead citrate (Scharlau, Germany). Microscopy was performed with JEM 1220 TEM (JEOL, Japan) microscope at 120-keV electron energy. To optimize the contrast, zero-loss energy filtering was applied [Reimer 1995].

RESULTS AND DISCUSSION

Morphostructural changes of cells of test bacterial strains (*S. aureus*, *L. monocytogenes*, *S.* Enteritidis, *E. coli*) caused by WEPE were visualized using TEM technique and are shown in the micrographs in Figures 1–4.

Untreated cells of *S. aureus*, *L. monocytogenes*, *E. coli* and *S.* Enteritidis in physiological saline showed a normal cell shape with undamaged and intact structure of the cell walls and well-defined membranes (Figs. 1A–4A). In case of Gram-positive strains the surface of cell walls was smooth and uniform. Cell divisions testifying to proper cell growth can be also observed (Figs. 1A–4A). The periplasmic space of Gram-negative bacteria was thin and had a uniform appearance (Figs. 3A–4A). Incubation cells with WEPE caused changes in cell morphology (Figs. 1B, C, D–4B, C, D). *Staphylococcus aureus* cells were deformed and showed dents (Figs. 1B–D). Membrane was shrunk and detached from cell wall (Fig. 1C). The cell wall rupture (Figs. 1D–E) and loss of structural integrity was observed (Figs. 1C–E), what result as cytoplasmic shrinkage and leakage of intracellular matrix (Figs. 1C–E). Similar effects include membrane shrinkage (Figs. 2B, D), cell wall rupture and cytoplasmic leakage (Fig. 2C) were also observed in case of *L. monocytogenes.* TEM micrographs of *E. coli* showed that treatment with cranberry extract results with swollen periplasmic space near the polar region of the cell (Fig. 3B), as well as the cells contain electron-dense material accumulated in the intracellular matrix (Fig. 3B). Continuity of outer and inner membrane was also disrupted and electron-dense material points were accumulated along the membrane (Fig. 3C). The influence of WEPE caused membranes disruption of some *S.* Enteritidis cells and forming additional condensed structures in them (Fig. 4B). Outer and inner membranes of other cells were intact,

but periplasmatic space was swollen, as well as periplasmatic space contains electron-dense material (Fig. 4C). Leakage of intracellular matrix was also observed and result as outer and inner membrane damage (Fig. 4D). Similar results were obtained by Wu et al. [2008] using *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus*. Cranberry concentrate caused similarly degenerative changes such as loss of cell integrity, cell deformation, cell wall rupture, as well as, cytoplasmic leakage and condensation.

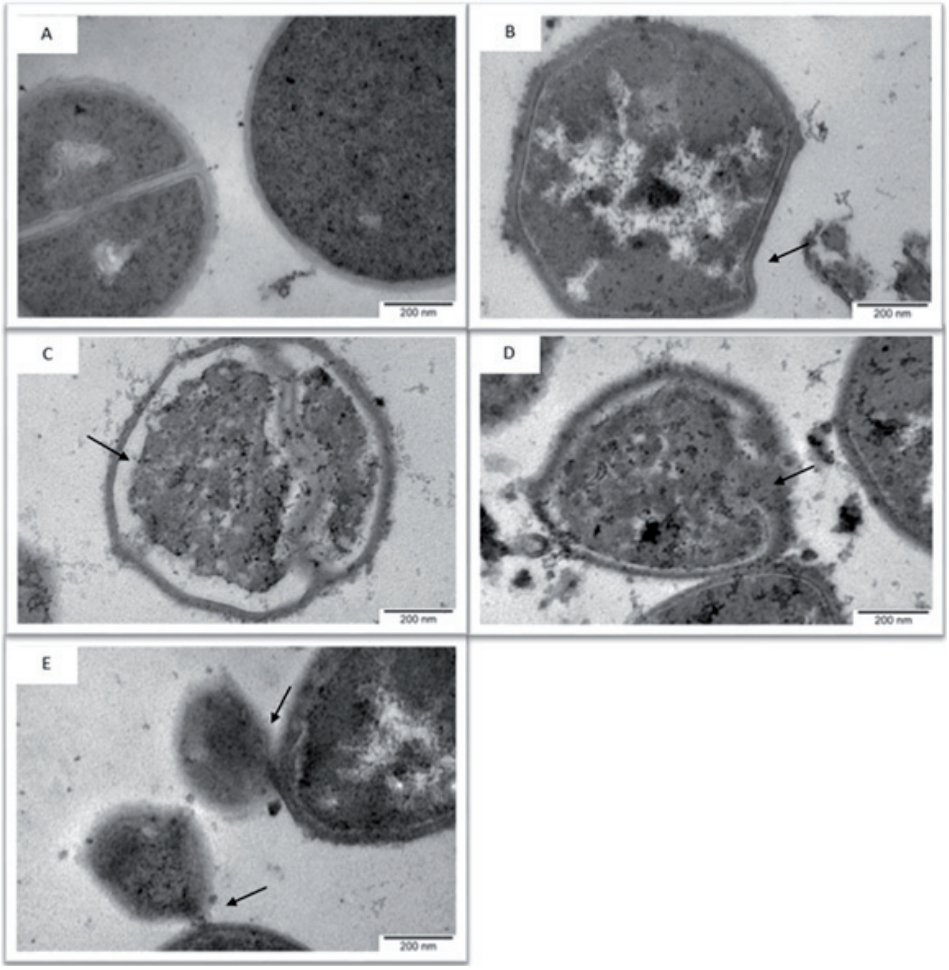

Fig. 1. TEM microphotographs of *S. aureus* A-529 untreated (A) and treated with cranberry extract (B, C, D, E). In physiological saline cell structure is smooth and continuous (A), while after treatment cells were deformed with dents (B), inner membrane detached from cell wall (C), cell rupture (D) and leakage of intracellular matrix (E)

Rys. 1. Mikrofotografie TEM komórek *S. aureus* A-529 z czystych kultur (A) oraz pod wpływem działania ekstraktu (B, C, D, E). W roztworze soli fizjologicznej struktura komórek jest zwarta (A). Pod wpływem działania ekstraktu komórki zostały zdeformowane (B), błona wewnętrzna uległa oderwaniu od ściany komórkowej (C), obserwowano pęknięcie komórki (D) i wyciek treści wewnątrzkomórkowej (E)

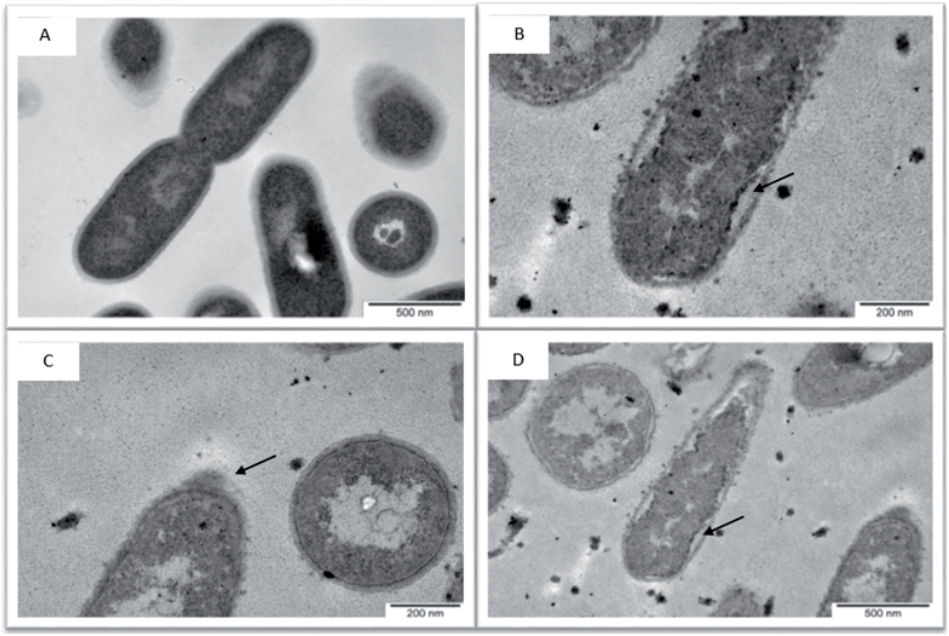

Fig. 2. TEM microphotographs of *L. monocytogenes* 17/11 untreated (A) and treated with cranberry extract (B, C, D). In physiological saline cells have intact structure (A), while after treatment cells indicated membrane shrinkage (B, D) and leakage of intracellular matrix (C)

Rys. 2. Mikrofotografie TEM komórek *L. monocytogenes* 17/11 z czystych kultur (A) oraz pod wpływem działania ekstraktu (B, C, D). W roztworze soli fizjologicznej komórki mają nienaruszoną strukturę (A). Pod wpływem ekstraktu obserwowano obkurczenie błony (B, D) i wyciek treści wewnątrzkomórkowej (C)

In our research, part of antibacterial activity of WEPE extract was probably a result of organic acids, however pH level in media with we-PSCE did not exceed the critical pH level required for the growth of tested strains [Puupponen-Pimiä et al. 2001]. According to Lacombe et al. [2010], phenolic compounds present in cranberry extracts, retain their antibacterial properties at neutral pH. Thus, the antimicrobial activity of WEPE is probably an effect of varied mechanisms of numerous natural bioactive compounds. Nevertheless, low pH creates an acidic environment outside, as well as, inside the cell, which increases the acetate and potassium ions concentration in bacterial cells. This situation results with the accumulation of organic acid anions on the bacterial cell membrane ultimately increased osmotic stress and lead to its destabilization [Lacombe et al. 2010].

Presumably, not only low pH, but also other mechanisms of action contribute to the antimicrobial effect of cranberry extracts. However, the mechanisms of antimicrobial activity of natural extracts still are not fully understood. There are some publications indicating the antimicrobial activity of organic acids, but the results are varied and differenced depending on the bacterial strains. Antibacterial mechanism of action of organic acids consists of their ability to penetrate cell membranes and their antibacterial activity increases with the decreasing degree of acid dissociation [Malinowska-Pańczyk et al.

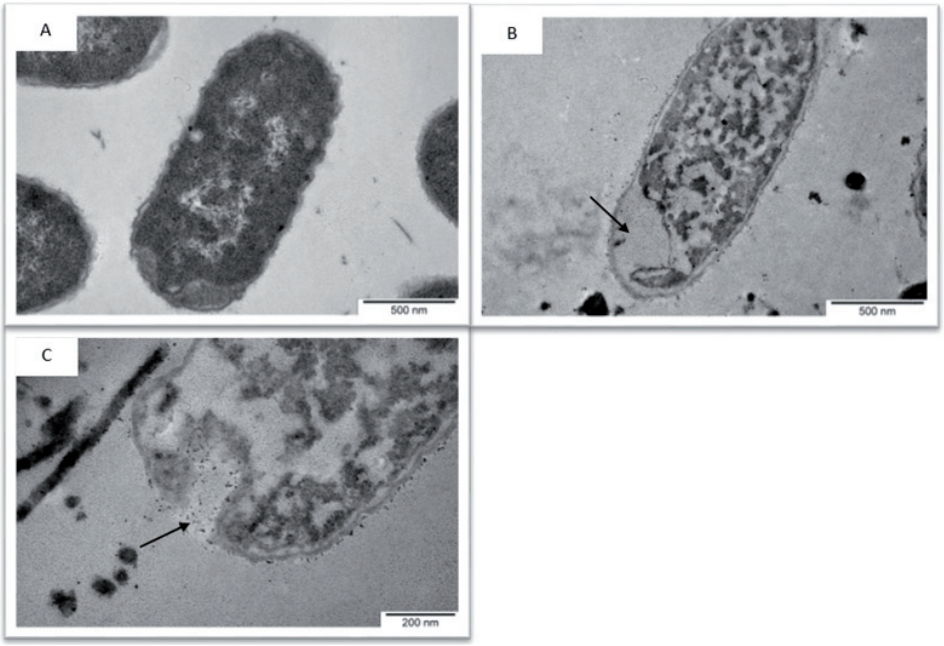

Fig. 3. TEM microphotographs of *E. coli* O26 untreated (A) and treated with cranberry extract (B, C). In physiological saline cell structure is smooth and continuous, the periplasmic space was thin with uniform appearance (A), while after treatment cells have swollen periplasmic space near the polar region of the cell (B) and cell disruption (C)

Rys. 3. Mikrofotografie TEM komórek *E. coli* O26 z czystych kultur (A) oraz pod wpływem działania ekstraktu (B, C). W roztworze soli fizjologicznej struktura komórek jest zwarta, a przestrzeń peryplazmatyczna cienka o jednolitym wyglądzie (A). Pod wpływem działania ekstraktu obserwowano powiększenie przestrzeni peryplazmatycznej komórki (B) i rozerwanie komórek (C)

2010]. Non-dissociated acid molecules may dissociate inside the cell, which results in lowering the cytoplasmic pH to a level lower than the tolerance limit. This process provokes removing protons from the cell, which requires a lot of energy affecting metabolism and, as a result, cell death. Antimicrobial activity may also depends on the physiological state of the bacterial strain, as well as, on the physicochemical properties of the external environment [Ricke 2003]. It is believed that the main mechanism plays a role in antibacterial activity is the ability of the antibacterial bioactive compounds to selective disruption of the bacterial cells cytoplasmic membrane, which causes potassium ion leakage and cell dysfunction [Yi et al. 2010, Lou et al. 2011]. Phenolic compounds can form complexes with proteins on the outside of the cytoplasmic membrane of bacteria by hydrogen and covalent bonds and by hydrophobic interactions, as well as changing the membrane potential of the Na^+/H^+, which reduces the cell tolerance in a low osmotic pressure environment [Haslam 1996].

The coffee phenyl ester, coffee acid and quercetin inhibit the mobility of fimbried bacteria (mainly Gram-negative strains) and cause cytoplasmic membrane permeability for metal ions.

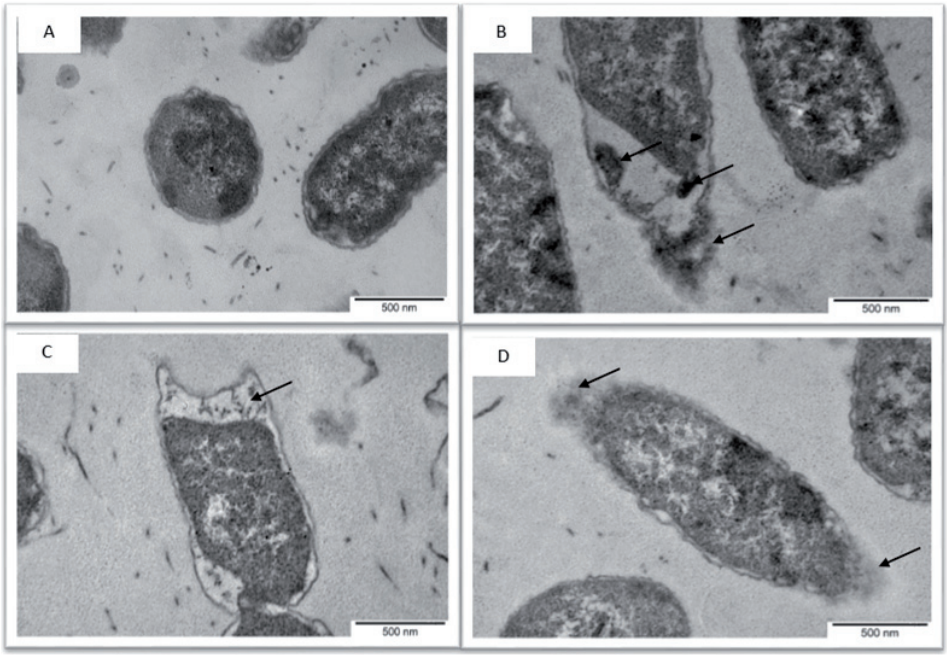

Fig. 4. TEM microphotographs of *S.* Enteritidis 322/11 untreated (A) and treated with cranberry extract (B, C, D). In physiological saline cell structure was well-defined and the periplasmic space was thin with uniform appearance (A). After treatment cells have disruption membrane with additional condensed structures in them (B), swollen periplasmatic space (C) and leakage of intracellular matrix (D)

Rys. 4. Mikrofotografie TEM komórek *S.* Enteritidis 322/11 z czystych kultur (A) oraz pod wpływem działania ekstraktu (B, C). W roztworze soli fizjologicznej struktura komórek była zwarta, a przestrzeń peryplazmatyczna cienka o jednolitym wyglądzie (A). Pod wpływem działania ekstraktu obserwowano pęknięcia błony i ściany komórkowej, wewnątrz komórki skondensowane struktury (B), powiększoną przestrzeń peryplazmatyczną (C) oraz wyciek treści wewnątrzkomórkowej (D)

According to Mirzoeva et al. et al. [1997], the caffeic phenyl ester, caffeic acid and quercetin inhibit the mobility of fimbried bacteria (mainly Gram-negative strains) and cause cytoplasmic membrane permeability for metal ions. Described action leads to the disorder of vital functions and the death of bacterial cells. As revealed in our study, antimicrobial compounds present in the cranberry extract damage the walls and membranes of bacteria and induce cell lysis and cellular leaks. These changes make it easier to access these compounds into the cell so that they can react with bacterial DNA and ultimately lead to cell death [Wu et al. 2008]. Puupponen-Pimiä et al. [2001] indicated the possibility of binding phenolic compounds from berry fruits to the outer cell membrane of Gram-negative bacteria, leading to disturbance of its permeability. Different cell wall structure of Gram-positive and Gram-negative bacteria may affect different types of cell damage under the same antimicrobial substance. The thick layer of peptidoglycan in the cell walls of Gram-positive bacteria provides them with greater rigidity compared to Gram-negative cell walls [Ghuysen and Hakenbeck 1994], which was confirmed in our results. Destruc-

tive changes involving total lysis and cell degeneration are primarily associated with Gram-negative strains. In the case of Gram-positive strains, the lesions consisted mostly of wall fracture, cell leaking and turgor loss. In addition, cells in the stationary phase appear to be less susceptible to the effect of the extract than the dividing cells, as was confirmed by Stewart et al. [2004].

CONCLUSIONS

Imaging with TEM confirmed antimicrobial activity of WEPE against foodborne bacterial pathogens. TEM micrographs of the extract-treated bacteria cells showed the evidence of changes in cell morphology like loss of structural integrity, wall and membranes rupture, shrinkage and leakage of cytoplasmic material and other damages which are evidences of cell lysis which clearly confirms its antibacterial activity. Obtained results give promising opportunities of using cranberry extract as a natural food preservative.

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USZKODZENIA MORFOSTRUKTURALNE KOMÓREK BAKTERII PATOGENNYCH PRZENOSZONYCH PRZEZ ŻYWNOŚĆ POD WPŁYWEM EKSTRAKTU Z ŻURAWINY BŁOTNEJ (*VACCINIUM OXYCOCCOS* L.) ZOBRAZOWANE ZA POMOCĄ TRANSMISYJNEJ MIKROSKOPII ELEKTRONOWEJ (TEM)

Streszczenie. Patogeny przenoszone przez żywność, gdy zanieczyszczają produkty spożywcze, stanowią poważne zagrożenie dla zdrowia ludzi. Obecnie konsumenci są zainteresowani naturalną, bezpieczną żywnością, a jednocześnie coraz bardziej zaniepokojeni stosowaniem chemicznych środków konserwujących i dodatków do żywności. Z tego powodu należy poszukiwać naturalnych alternatywach metod konserwowania produktów

spożywczych. W piśmiennictwie dostępne są dane dotyczące właściwości przeciwdrobnoustrojowych owoców żurawiny, jednak informacje na temat uszkodzeń morfostrukturalnych komórek patogennych bakterii pod wpływem ekstraktów żurawinowych są nadal ograniczone. Niniejsze badania zostały przeprowadzone w celu oceny zmian morfostrukturalnych pod wpływem wyciągu z wodno-etanolowego ekstraktu z wytłoków żurawiny błotnej na wybrane bakterie patogenne przenoszone przez żywność, w tym Gram-dodatnie (*Staphylococcus aureus* i *Listeria monocytogenes*) i Gram-ujemne (*Eschericha coli* i *Salmonella* Enteritidis). Zmiany morfologiczne i strukturalne komórek bakterii poddanych działaniu ekstraktu zaobserwowano za pomocą techniki transmisyjnej mikroskopii elektronowej (TEM). Przeprowadzone badania wykazały zmiany w strukturze komórkowej i integralności komórek zarówno dla szczepów Gram-dodatnich, jak i Gram-ujemnych. Stwierdzono znaczne nieodwracalne uszkodzenia strukturalne komórek takie jak pęknięcie błony komórkowej wraz z wyciekiem treści wewnątrzkomórkowej. Obecne wyniki wskazują, że badany ekstrakt jest wysoce skuteczny przeciwko patogenom przenoszonym przez żywność, co wskazuje na obiecujące potencjalne zastosowanie ekstraktu z żurawiny jako naturalnego środka konserwującego w produktach spożywczych. Wpływ badanego ekstraktu na inne procesy komórkowe, takie jak: hamowanie syntezy kwasu nukleinowego, hamowanie syntezy ściany komórkowej i białka błonowego lub zaburzenie metabolizmu energii komórkowej, powinien być przedmiotem dalszych badań.

Słowa kluczowe: wytłoki z żurawiny, aktywność antybakteryjna, TEM, zmiany morfostrukturalne komórek, patogeny bakteryjne