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Research on Thermal Imaging Usage as a Method for Detecting *Bacillus subtilis* Bacteria in Mortadella

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Abstract: Meat and meat products are important sources of valuable proteins and other nutrients, but they are also a conducive environment for microorganisms' growth and can easily corrupt. In order to ensure the safety and quality of these products throughout its entire shelf-life, reliable microbial contamination assessment techniques must be used. Despite their effectiveness, traditional diagnostics methods are expensive and time consuming. Therefore, new timesaving, reliable techniques are searched for. Infrared thermography could be a good alternative method in this area. As a quick technique to detect microorganisms, it can overcome the limitations of traditional microbiological food-quality assessment methods. It has numerous advantages, such as the possibility of taking immediate temperature measurements and short processing times for obtaining a thermal image, non-contact and non-destructive measurements, and, unlike other methods, real-time measurement monitoring. Real-time monitoring is particularly important for modern production systems. The purpose of this research study is to develop a methodology for microbiological quality control of mortadella inoculated with *Bacillus subtilis* ATCC 6633 bacteria using a thermal imaging camera with an uncooled microbolometric detector. It was found that the thermal imaging measurements used in this research study enabled the distinction of contaminated samples (min. 10^6 CFU/g) from sterile samples. The tests should only record the temperature of the samples during the first 25 s after previously performing activations at −18 °C ± 1 °C for 60 s. This is where differences between the samples are most pronounced. Estimating the trend line of the sample's cooling process is advisable.

Keywords: thermal imaging camera; microbiological quality; mortadella; *Bacillus subtilis*

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1. Introduction

The microbiological contamination of food products is a serious issue not only because of the security risk but also because of economic losses related to utilizing unfit food [1]. Conventional techniques are used most often when identifying and quantifying microorganisms. Their applications are laborious, take a lot of time, and most importantly require qualified personnel. Therefore, research is conducted to develop rapid techniques for the microbiological quality analysis of food products [2]. In this regard, innovative methods, including thermal imaging, are becoming more and more popular.

The infrared thermography method (IRT) is based on the observation of any object at a temperature above absolute zero (−273.15 °C) and recording the radiation distribution in the infrared range. Infrared radiation emitted by the tested surface is recorded with a thermal imaging camera, which transforms radiation into an image called a thermogram. The obtained image illustrates temperature distributions on the tested surface. It is presented in the form of colored isotherms, where the points of the same temperature have one color. What is important is that the measurements performed with infrared cameras can be conducted in the dark because the thermogram shows each object that emits infrared radiation [3,4].

The research using thermal measurements can be divided into two groups: passive and active thermal imaging. In passive imaging, the thermography method-tested object

characterizes only the temperature field created during its functioning. In active methods, additional sources of thermal stimulation—heating or cooling—are used. By thermodynamics, both methods of thermal stimulation are equally valuable, with identical powers with respect to the thermal radiation stream. Considering the density of heat streams and the possible obstacles or interruptions, heating is more practical [5].

Initially, thermal imaging was developed for military use. The first research study reporting its usage was limited due to the low sensitivity of thermal imaging systems that were available at that time. With the development of high-resolution digital imaging in analytical tools, thermal imaging measurements started to be used in various domains, such as medicine, material science, fire security [6], veterinary, aviation [7], energetics, construction, metallurgy, and in scientific research on thermodynamic phenomena and heat exchange [8].

Thermal imaging could be also used in the quality assessment of agricultural products and in so-called post-harvest operations. The research contains fruit, vegetables, and meat quality assessment; foreign body detection; cooked food temperature mapping; or packaging flaw detection. Thermal imaging can be also used in all locations that require controlling product temperatures on the production line (e.g., ready-to-eat food production) [7,9–11].

While working on a easy, fast, and sensitive microbiological diagnostics technique that could eliminate the preparation and sample modification phase, Lahiri et al. [12] noticed that all living organisms, including bacteria, produce heat as a result of basic metabolic processes. This emitted thermal energy can be utilized to detect microorganisms using infrared thermography. It was observed that the energy (EC), defined as a ratio of heat created by bacterial metabolism to heat lost, linearly increases with an increase in pathogenic bacteria concentrations (*Vibrio cholerae*, *Vibrio mimicus*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*) [12].

In other research using thermal imaging cameras [13], *Escherichia coli* bacteria contamination was detected. The results showed 100% effectiveness of the examination and the minimum time required for microbiological contamination detection was 5 h. Salaimeh et al. [14] also realized the potential of thermal imaging techniques with respect to *E. coli* bacteria quantity indication. In this case, infrared images (video IRT), thermodynamic laws, and heat exchange mechanisms (convection, conduction, and radiation) were used in order to perform direct, real-time measurements of energy loss from heat from the surface of the sample containing living cells while cooling for 2 min. This technique takes living cell features into consideration and distinguished them from dead or metabolically neutral cells and can be used to count bacteria in water and other fluids. The above research assumes that bacteria's energy production speed increases as a result of their metabolic activity and transformation from complex biochemical compounds into simpler forms [14].

A calorimetric analysis of absolute anaerobe *Clostridium acetobutylicum* growth confirmed that heat emissions correspond to the microorganisms' cell concentration, so heat emissions should double since the number of microorganisms cells doubled [15]. It is also assumed that the presence of bacteria and their metabolic activity can influence the thermophysical and optic features of the nutrient material, e.g., its emissivity. The positive value of energy (EC) is a result of exothermic reactions and indicates the tested sample's temperature or an increase in temperature with respect to the control sample. The negative value of energy indicates endothermic reactions and a decrease in temperature or emissivity [14].

Thermography as a quick technique for the microorganisms' detection could potentially overcome the limitations of conventional microbiological food-quality assessment methods. It has numerous advantages, such as the following: the fact that it is non-destructive (it can be used to test samples in a non-invasive manner), non-contact measurements that prevent food contamination risk, the possibility of instantaneous temperature measurement for the chosen point, short processing times to obtain a thermal image, the lack of harmful radiation during measurements, and the ease of use and low complexity in

tooling. Contrary to other methods, thermal imaging allows for the simultaneous monitoring of large surface (high number of samples at the same time) and temperature changes in real time [9]. However, before it can be commonly used as an online tool for food quality assessment, several limitations must be overcome. The challenge with the mass usage of thermal imaging sensors includes, among others, the need to cool or heat the product in order to increase the temperature's contrast, thermal disturbance from the environment, the heterogenous background of the examined object (as in the case of belt conveyors systems), and the existence of dead pixels in the detector [6]. Other issues that can occur are changeable lightning conditions causing background disturbance that can be observed on the thermograms and the fit of existing algorithms and optimal filter combinations with respect to various kinds of food products. If future research will provide a solution to these problems, the microorganisms' detection using thermal imaging can become a standard procedure ensuring the security of food at mass production scales [1].

This study was an attempt to develop a method for *Bacillus subtilis* contamination growth monitoring in mortadella using thermal imaging.

2. Materials and Methods

2.1. Materials

Mortadella in its bar form was purchased in the local hypermarket and was used as the test material. *Bacillus subtilis* ATCC 6633 bacteria strains from the American Type Culture Collection (Manassas, VA, USA) were reproduced in the probe in agar PCA (with yeast extract, glucose and casein peptone, Merc Millipore) for 24–48 h in 37 °C. The inoculum was prepared in 0.85% NaCl in order to obtain the level of 1×10^8 CFU/mL (Densimat, bioMérieux Italia S.P.A) [16]. Then, the obtained suspension was diluted to the following levels: 10^7, 10^6, and 10^5 CFU/mL.

2.2. Mortadella Samples Preparation for the Study

Mortadella was cut into slices of 0.5 cm in width and 5.5 cm diagonally and placed on a Petri dish. The prepared product was sterilized in 121 °C temperature for 15 min. Samples were contaminated on the surface using a cell spreader with a 0.5 mL appropriate suspension of bacteria (10^8, 10^7, 10^6, and 10^5 CFU/mL) and by incubating at 22 °C temperature for 24 h. After incubation, bacterial counts were performed for each sample using a plate culture method on PCA (incubation at 37 °C for 48 h). At the same time, the samples were examined with a thermal imaging camera, which allowed for the comparison of the results from the classical microbial counting method with the thermal imaging method. For each series of tests, the following set of samples was prepared: control sample (uncontaminated) and three samples for each of four contaminations variant. All data were collected in a single experiment.

To measure radiation emissions, a computer-connected camera, KTL-10 (Etronika Sp. z o.o., Poland), with an uncooled microbolometric detector ($640 \times 480 - 17$ µm) was used. The temperature distribution was registered using the active thermographic method. Contaminated and sterile samples were kept (activated) for 60 s at -18 ± 1 °C, and right after that, thermal imaging camera measurements were performed every 5 s and lasted for 2 min. The device was installed on a tripod (15 cm above examined sample) in order to obtain thermograms of the highest quality. Image sharpness was adjusted manually by setting the lens. In order to eliminate any disturbances caused by heterogenous backgrounds, samples were placed under the camera on a white surface. To compare the thermogram with actual sample appearances, digital photos were made in addition to thermal imaging.

Thermovision research was performed under established conditions as follows: humidity at 27 ± 1% and temperature at 23 ± 0.1 °C (AURIOL HG04705, Langenzenn, Germany). After the measurements were concluded, the results were obtained in the form of thermograms with the indicated temperature value at the central point of the mortadella sample. The process of thermogram creation in thermal imaging camera is as follows:

the image of the examined object placed upon the matrix generates electrical signals via microdetectors. Its strength depends on the wave length and radiation intensity. This signal is then pushed to the processing and visualization unit to finally create a thermogram [5].

2.3. Statistical Analysis

To perform statistical analysis, Statistica 13.3 (TIBCO, Palo Alto, CA, USA) and Microsoft Office Excel 2016 (Microsoft Corporation, USA) programs were used. For the obtained measurement results, arithmetic means and standard deviations were determined. Diagrams showing the temperature of activated samples and its change over time were created. The trend line and straight-line equation were adjusted along with the R^2 coefficient of determination and error bars with the standard deviation. Microsoft Office Excel 2016 was used for the above-mentioned analysis. In order to determine temperature variabilities between different degrees of sample contamination, the directional coefficients, a, of the equations determined for the trend lines were compared using Statistica 13.3 software. The directional coefficient, a, determines the linear function's inclination angle to the *x*-axis. A single factor variance analysis (ANOVA) carried out using Tukey's was utilized to establish the significance of differences between the control samples and various contaminated samples and to divide samples into homogeneous groups. Differences were marked as significant if the *p*-value is <0.05.

3. Results and Discussion

Previous research on the field of measurements of the thermal energy produced by microorganisms proves the feasibility of using a thermal imaging camera for their detection [12,14,15,17,18]. The amount of emitted heat has been shown to increase with the number of bacteria [15]. A mathematical relationship between heat generation and microbial growth has been found [17]. However, research on the development of a methodology for detecting microbial contamination using thermal imaging is still underway. On this basis, it was considered advisable to conduct research in this area. The research used bacterial strain *B. subtilis*, because the spoilage of meat foods is often a result of the growth of these bacteria [19]. *B. subtilis* causes ductility, is slimy, and induces an ammonia-musty smell in meats product. In order to maintain good quality products throughout its entire shelf-life and reduce food waste, reliable microbial contamination assessment techniques must be used. Despite their effectiveness, traditional diagnostics methods are expensive and time consuming. Therefore, with respect to infrared thermography as a quick technique for detecting microorganisms, it can overcome limitations of traditional microbiological food-quality assessment methods.

The number of bacteria in the contaminated samples that were incubated at 22 °C for 24 h is shown in Table 1.

Table 1. Determined number of bacteria in the tested mortadella samples (mean values ± standard deviation).

Sample	Inoculation (CFU/mL)	Bacteria Number (CFU/g)
Control	-	Absent in 0.1 g
1	1.0×10^5	$6.8 \times 10^6 \pm 2.1 \times 10^5$
2	1.0×10^6	$5.2 \times 10^7 \pm 1.1 \times 10^6$
3	1.0×10^7	$9.5 \times 10^8 \pm 2.3 \times 10^7$
4	1.0×10^8	$2.2 \times 10^9 \pm 5.4 \times 10^7$

At the same time, for contaminated samples such as *Bacillus subtilis* ATCC 6633 bacteria, tests were performed using a thermal imaging camera. Figure 1 represents the images of contaminated samples with 6.8×10^6 CFU/g bacteria, which were recorded at 15, 45, 74, and 105 s.

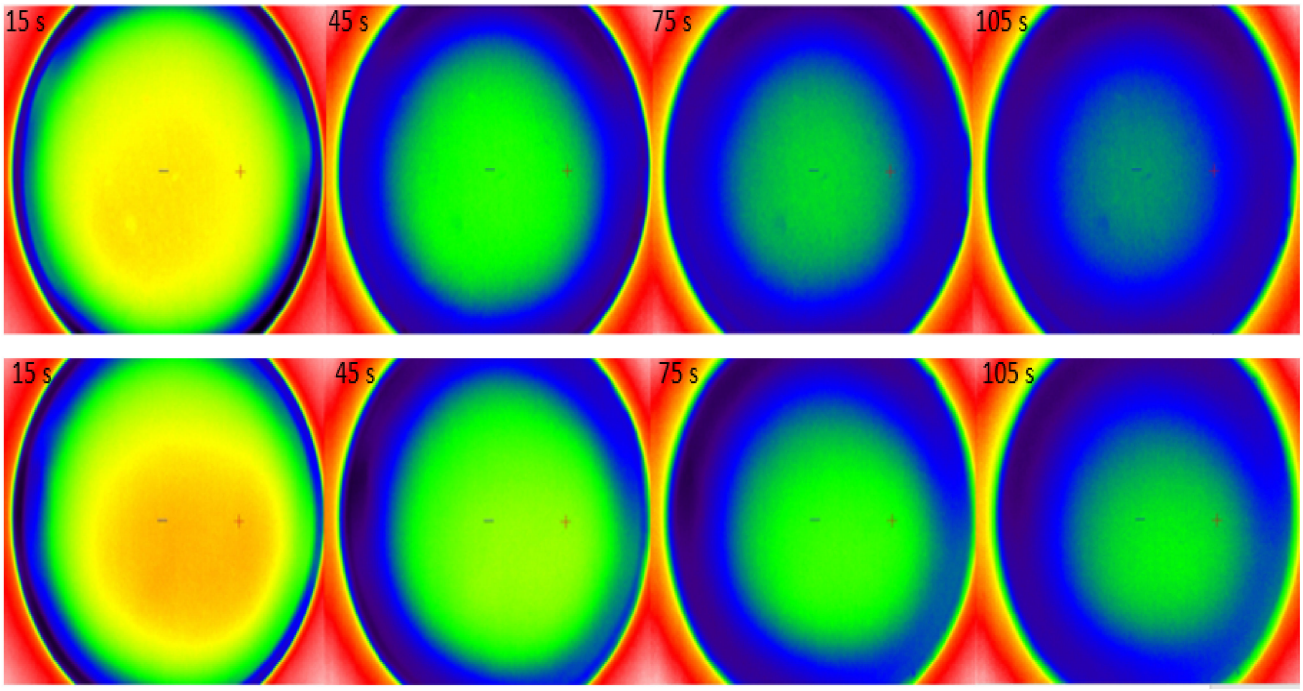

Figure 1. Thermograms of mortadella samples thermograms at 15, 45, 75, and 105 s with the thermal imaging camera. Upper row: Uncontaminated sample (control). Lower row: Sample contaminated on the surface with a *B. subtilis* ATCC 6633 suspension with 6.8×10^6 CFU/g contamination degrees.

In the above thermograms, one can see changes taking place in time. The samples cooled down and the background (the plastic dish) is slightly heated. The highest temperatures on the sample's surface, visible in orange and yellow, registered in the most central places and the lowest temperatures registered at the boundaries. The heat loss speed difference between the sterile control sample and the sample contaminated with the *B. subtilis* ATCC 6633 suspension was observed. Salaimeh et al. [14] had similar observations. They monitored changes in temperature in a liquid environment with various contamination degrees using infrared thermography. They observed a drop in temperature from 29.68 °C to 20.88 °C at the end of 2 min during their measurements. The temperature of the samples containing bacteria in the range of 120 to 5.0×10^8 CFU/mL decreased from 29.48 °C to 21.05–23.7 °C at the end of the measurement period depending on the bacterial concentration in the sample. When the liquid environment cooled, the differences in temperature between the control and contaminated samples increased within 120 s of conducting measurements for all variants except for the ones that included less than 6.0×10^3 CFU/mL.

Figure 2 shows the distribution of temperature changes in mortadella samples with various degrees of contamination and the sterile sample after its activation.

In diagrams of the contaminated samples (2b–e), two trend lines were recognized: for temperatures, changes between 5 and 25 s and between 25 and 120 s upon measurement. It was necessary to recognize two trend lines to differentiate the sterile and microbiologically contaminated samples in the shortest period of time.

Linear functions fit the model very well, which was indicated by high values of coefficient R^2 (from 0.95 to 0.99). During the first 25 s of measurement, a quicker loss of heat was observed, which was indicated by the steeper course of the linear function. At 25 s, the sample's cooling speed significantly dropped—the diagram became flatter. No divisions into two parts were observed only for the control sample's results, and one trend line was assigned, which is also described by a linear function with the fit factor R^2 equal to 0.9034. In the research conducted by Gardea et al. [17] on the calorimetric assessment of microorganisms' growth in milk, a mathematical correlation between created

heat and microorganisms' growth was also found; however, it was defined by a third-order polynomial ($R^2 = 0.99$).

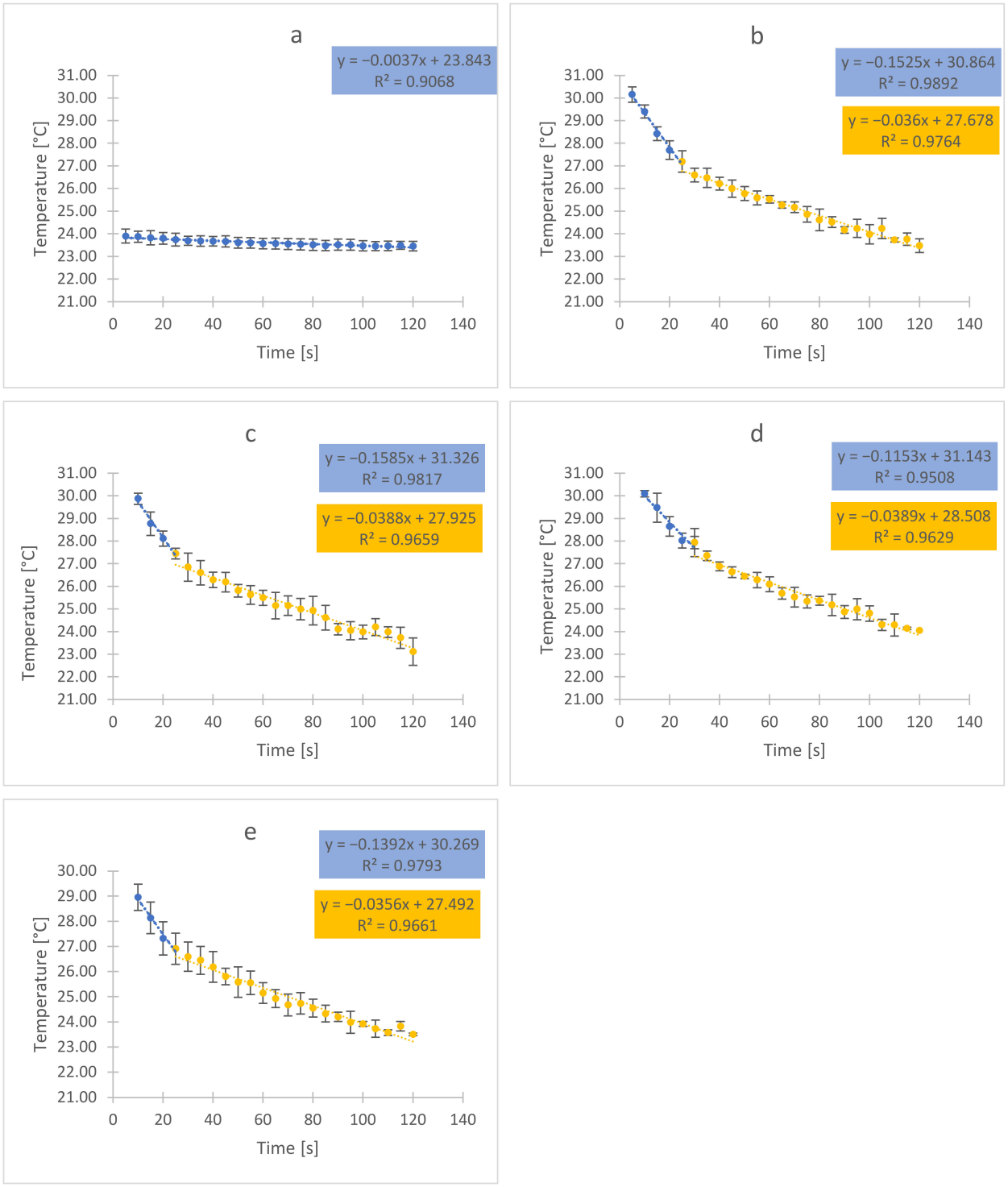

Figure 2. Distribution of temperature changes in mortadella samples after activation (mean values and standard deviation). From the top: Changes in control sample temperatures (**a**) and for samples contaminated with the *B. subtilis* ATCC 6633 suspension with the following densities: (**b**) 2.2×10^9 CFU/g, (**c**) 9.5×10^8 CFU/g, (**d**) 5.2×10^7 CFU/g, and (**e**) 6.8×10^6 CFU/g. The temperatures were registered for 2 min every 5 s. Blue represents the trend line for temperature changes between 5 and 25 s and orange represents trend lines between 25 and 120 s of the measurement.

In order to determine temperature variabilities between different degrees of sample contamination, the directional coefficients, a, of the equations determined for the trend lines were compared. In Figure 3, one can see the calculated slope factors of a in the trends assigned for samples with different contamination degrees and the control sample. For each variant, the negative values of coefficients were obtained, which indicates that, as time passes, the temperature lowers.

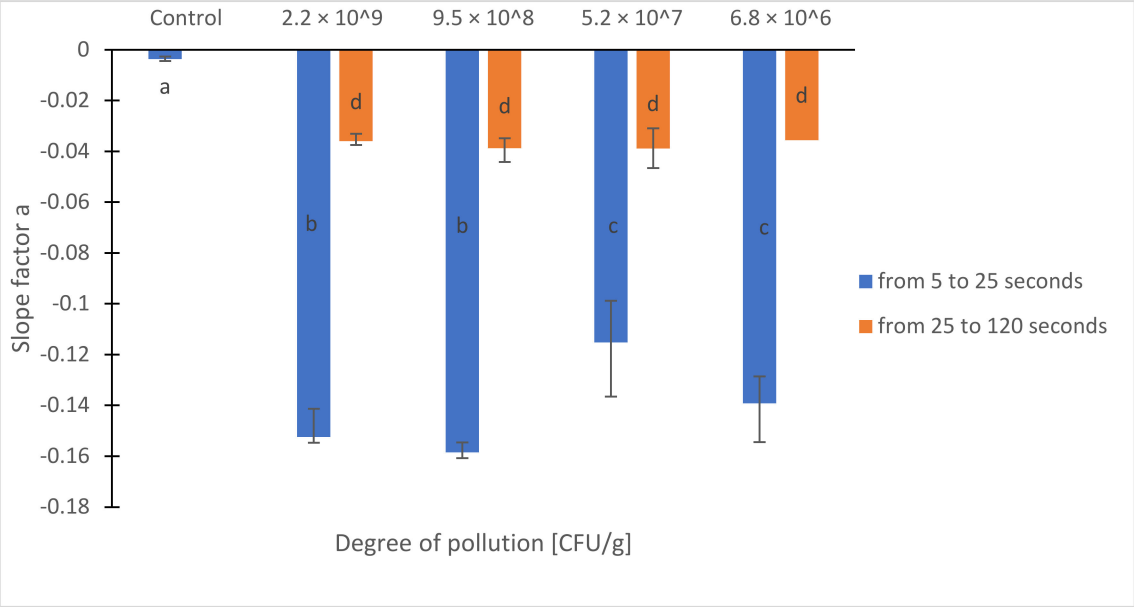

Figure 3. Comparison of temperature regression coefficients (mean values and standard deviation) relative to the time after the activation of *B. subtilis* bacteria contamination samples: 2.2×10^9 CFU/g, 9.5×10^8 CFU/g, 5.2×10^7 CFU/g, and 6.8×10^6 CFU/g; a–d—different letters on the bars indicate statistically significant differences between the samples by Tukey's test ($p < 0.05$).

The slope factor, *a*, for sterile (control) sample was significantly lower in comparison to the remaining samples. This implies that the uncontaminated sample loses heat slower. By analyzing the distribution of temperatures measured before 25 s and after 25 s for the contaminated samples, it was observed that, in the initial phase, the slope factor, a, of the obtained trend line was higher for each variant. The highest speed of heat loss was registered for samples with a contamination degree of 9.5×10^8 CFU/g and 2.2×10^9 CFU/g. The samples with contaminations equal to 6.8×10^6 CFU/g and 5.2×10^7 CFU/g cooled slower. Based on cooling dynamics, they can be easily distinguished from other variants of contamination, especially from the control sample. Usually, the number of microorganisms lower than 10^6 CFU/g does not result in visual changes in the meat product, so these results can open the possibility to discover corruption before it is even noticeable. However, there is a relation observed by Miyake et al. [15] in their calorimetric study on anaerobic microorganisms *C. acetobutylicum* and *C. beijerinckii* growth. They claimed that the amount of created heat was proportional to the number of microorganisms, and they revealed the existence of a very good correlation between heat emission and optic density/ATP concentrations. The reason for obtaining different observations can be physiochemical changes taking place in corrupted mortadella samples. The change in the structure can contribute to an increase or decrease in the sample's surface emissivity, which can cover up the emissivity of microorganisms themselves and influence the results of measurements using thermal imaging cameras. After 25 s of measurements, no significant differences between heat emitted by tested samples with different contamination degrees were registered. This result suggests that, in the following research, the temperature of samples should be registered only in the first seconds of cooling after a previous activation since the differences between contaminations are then best visible. What is also interesting is the

behavior of the mortadella sample after activation in −18 ± 1 °C. Before the research study, it was assumed that the samples taken out of the freezer will gradually heat up by using heat from the environment. However, they showed an opposite tendency, meaning that despite being moving from lower to higher temperatures, they kept losing heat. It could be caused, among others, by the accumulation of heat in multiplied biomass in tenderloin samples, which lost heat to the environment faster than the mortadella itself. The results of Salaimeh et al. [14] confirmed that emissivity is dependent on, among others, the type and morphology of microorganism cells. It was suggested that the presence of bacteria and their metabolic activity can influence the optic and thermophysical properties of food materials, including its emissivity.

Gardea et al. [17] established a mathematical dependency between microorganisms' growth and heat emission. The results of these study also indicate the possibility of detecting heat related to *B. subtilis* bacteria metabolism. The temperature changes distributions in times of 2 min after the activation can be expressed by two linear functions with divisions into two time periods, between 5 and 25 s and 25 and 120 s, which were indicated by high fit factors, R^2. It is not possible to precisely determine the contamination degree by using the camera with an uncooled microbolometer, which was utilized in this study. What is satisfactory is that there are differences in heat loss speeds, which allows the determination of samples in which microorganisms are not present in and allows indicating contaminated samples, among others, in the degree of 10^6 CFU/g. These results show that measurements could proceed in a short period of time toward 25 s after previously performed activations at −18 ± 1 °C for 60 s. This is where the differences between samples are most pronounced. The concept presented is a proposition of new quality methods, which could be excellent alternatives to classical methods. It seems that it is easier to take a sample and to activate it at temperatures of −18 °C while using a thermal vision camera compared to using classic microbiological research methods in food industries. Many preliminary research studies have proved the possibility of using thermovision methods in detecting microorganism pollution in mortadella. In future studies, we plan to conduct additional analyses to improve the thermal imaging process for detecting microorganism growth.

These results show that the thermal imaging technique has high potential as a tool for monitoring microbiological food quality. However, before it can be commonly used, several issues with respect to the precision of results must be solved. The challenge with the thermal imaging sensors developed for microbiological research includes, among others, the need to cool or heat the product in order to increase the temperature's contrast, thermal disturbance from the environment, the heterogenous background of the examined object (as in the case of belt conveyor systems), and the existence of dead pixels in the detector [6]. Per Teena et al. [1], another issue that can occur includes changeable lightning conditions causing background disturbances and inconvenient reflections that can be observed in the thermograms and fitting existing algorithms and an optimal filter combination fit to various kinds of food products.

4. Conclusions

The study showed, based on the mortadella example, that thermal imaging cameras can be used as tools for monitoring microbiological changes happening in animal products. The active thermography based on the sample cooling for 60 s at −18 °C ± 1 °C used in this study emerges as a good fit for microbiological food quality assessments since it provided contrasts between the tested sample and the background and showed a clear difference in heat emissions between them. It was shown that the thermal imaging method allows distinguishing samples with absent microorganisms from the contaminated samples, i.e., including a minimum of 10^6 CFU/g *Bacillus subtilis*. The contaminated samples showed lower speeds of heat emission in comparison to the samples with 10^6 to 10^9 CFU/g degrees of contamination. The measurements using thermal imaging camera have the potential of utilization in monitoring the quality of microbiological food products. However, it is

necessary to conduct further research in order to enable distinguishing various degrees of contamination based on the microorganism's heat emission.

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