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Determination of *Escherichia coli* in Raw and Pasteurized Milk Using a Piezoelectric Gas Sensor Array †

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Abstract: The importance of assessing the microbiological safety of food products is beyond doubt, which is also true for milk and dairy products. The goal of this work was to evaluate the changes in the composition of the gas phase in milk based on signals from chemical sensors to predict the quantity of the bacteria in the milk samples. The gas phase in raw milk samples and samples during pasteurization, as well as for a standard (a model aqua solution of macronutrients and minerals), was studied using an array of sensors with polycomposite coatings, including those contaminated with *E. coli* bacteria. Assessment of microbiological indicators was carried out according to GOST in parallel with the gas-phase analysis. The applicability of the results obtained on model systems was assessed using milk samples, including those containing other types of pathogenic microorganisms (*Staphylococcus aureus*, *Klebsiella* spp., etc.). It was found that the obtained models can be used to assess the presence and quantity of *E. coli* in milk at the pasteurization stage.

Keywords: sensors; *E. coli*; contamination; cow's milk; pasteurization

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1. Introduction

Milk and dairy products have a priority importance in the diet of the population, since they contain all the nutrients necessary for the human body (proteins, fats, carbohydrates, minerals, vitamins, water) in well-balanced proportions and in an easily digestible form [1]. On the other hand, the macro- and microcomponents of raw milk are available sources of carbon and energy; therefore, when combined with the high activity of water, they can cause rapid native and extraneous microorganism growth [2].

Pathogenic microorganisms (*Campylobacter jejuni/coli*, *Escherichia coli*, *Listeria* spp., *Salmonella typhi* serotype, *Salmonella typhimurium* serotype, *Shigella* spp., *Staphylococcus aureus*, *Clostridium botulinum/perfringens*, *Streptococcus pyogenes*, *Vibrio cholerae*, etc.) are a serious problem in the processing of raw materials from animal origin, including milk, because they cause numerous types of food poisoning and diseases up to a fatal outcome, and they can also lead to the return of finished products from stream of commerce. Nowadays, the annual economic damage from mortality, diseases and their chronic consequences,

disability, and product return from the markets is estimated at several billion dollars [3,4]. In this regard, the control of the presence of pathogens in the raw materials and during the technological process is a primary task of processing enterprises.

For example, *E. coli* belongs to the *Enterobacteriaceae* family and these are part of the intestinal microbiota in warm-blooded animals. They enter the environment from feces, including onto the hands of farm workers, and can contaminate water and soil, and enter raw milk during milking. Most *E. coli* species are non-pathogenic, but they are considered sanitary indicative, because their presence or absence is used to assess the observance of sanitation and hygiene in the production of raw milk. However, many of them are capable of mutation. For example, enteropathogenic *E. coli* has evolved into a new species of *E. coli O*157:*H*7, which has caused numerous diseases due to food poisoning, such as gastroenteritis, dysentery, urinary infections, sepsis, pneumonia, and meningitis when ingested by humans [5]. Therefore, the control of pathogens in food raw materials is currently receiving great attention, including reducing the pathogen load even before the raw materials reach the processing plant. In this case, methods for express control of such microorganisms' presence, allowing for a reduction in the duration of microbiological analysis from several days to several minutes, are becoming important. They consist of the analysis of intracellular compounds and components of the microorganisms' cell wall or their indicators released during the growth or metabolism of bacteria. These methods include the detection of bacteria by bioluminescence of the generated adenosine triphosphate (ATP) [6], by the DNA structure of their cells using polymerase chain reaction [7], or loop isothermal amplification of nucleic acids [8,9], as well as chemical or biochemical recognition of various microorganisms' metabolic products using sensors. In the development of sensory methods for detecting food pathogens, there are trends toward increasing the sensitivity of DNA- and antibody-based biosensors [10,11], as well as the use of new compounds (peptides, aptamers, nanomaterials) to form sensitive biosensor coatings [12,13]. In particular, various biosensors based on aptamers [14,15], bacterial cells and their proteins [16], and antimicrobial peptide [17] are proposed for detecting coliform bacteria in milk. Gas chemical sensors capable of recognizing volatile metabolites of various microorganisms are also used to detect and monitor microorganisms in milk and dairy products. Such sensors are successfully used to predict shelf life, detect microbial spoilage, control product quality by detecting foreign tastes, and determine the total microbial count [18,19]. However, the problem of differential detection of pathogenic microorganisms in milk, including *E. coli*, remains unsolved; such studies are few in number [20]. A more detailed comparison of various methods for determining pathogenic microorganisms in milk is presented in the review [21]. Piezoelectric quartz sensors are frequently used in food analysis. Their operating principle is based on the attenuation of a volumetric acoustic wave during the sorption of components on the surface of an electrode glued to an AT-cut quartz crystal. Since the final characteristics of the sensors are highly dependent on the transducer, the comparative characteristics of various types of sensors are presented in Table 1 [22–26].

The aim of this work was to evaluate the change in the composition of the gas phase of milk based on signals from chemical sensors when it is contaminated with *E. coli* in order to develop a method for predicting the content of bacteria of the coliform bacteria group in the technological process of obtaining fermented milk products.

Table 1. Comparative characteristics of various types of sensors.

Type of Sensor	Limit of VOC Detection	Advantages	Disadvantages
Chemoresistive	5–500 ppm	High sensitivity, low operating temperature, and a thermal stable structure, simplicity, low cost, small size, and ability to be integrated into electronic devices	High sensitivity to water vapor, high possibility of sensor poisoning, low selectivity
Optical	1 ppm–1000 ppb	Commercial availability, simplicity of sensor formation	The complexity of creating devices, fluorescent dyes have a short operating time
Metal oxide	1–1000 ppm	Low power consumption, the possibility of long battery life, long life of the sensor material, ability to work in explosive environments	Low selectivity, poor sensitivity to organic molecules and relatively low stability caused by recrystallization and surface poisoning processes
Piezoelectric quartz microbalance	10 ppm–10 ppb	Linear calibration curve over a wide concentration range, fast response and recovery time, high sensitivity	Fragile sensing element, possibility of electrode corrosion
Surface acoustic waves (SAW)	1 ppm–1 ppb	High sensitivity, excellent response time, small size, low cost, ability to work in wired and wireless mode	Membrane aging

2. Materials and Methods

To study changes in the gas phase of milk in the presence of microorganisms, changes in the gas phase in control samples were preliminarily studied. As control samples, two variants of reference milk samples were selected as a result of sequential mixing of components (cream with a fat content of 20%, Avida OJSC, Moscow, Russia; milk protein concentrate, branch of PJSC MK Voronezh Cheese Factory Kalacheevsky, Voronezh, Russia; anhydrous lactose, Lenreaktiv OJSC, Saint Petersburg, Russia; calcium lactate 5-aqueous, Vekton OJSC, Russia; calcium dihydrogen phosphate 1-aqueous, Vekton OJSC, Saint Petersburg, Russia; dry skim milk, branch of PJSC MK Voronezh Kalacheevsky Cheese Factory, Russia) after preliminary dissolution of dry ingredients in distilled water. The composition of reference sample No. 1, composed of individual components present in raw whole milk: fat content (4.0–4.1)%; protein content (3.0–3.1)%; lactose content (4.65–4.70)%; mass fraction of ash (as a mixture of calcium lactate and calcium dihydrogen phosphate) (0.60–0.65)%; mass fraction of dry matter (12.3–12.5)%.

Further, the reference samples were contaminated with *E. coli* bacteria, which are the most frequently occurring microorganisms in the milk microflora, leading to defects in the appearance of milk and finished products, in addition to gastrointestinal diseases in humans.

The pure culture of *E. coli* was revived by culturing on an orbital shaker at 30 °C and 170 rpm in LB nutrient medium (peptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L; pH 7.0) for 20 h. The liquid suspension was plated in Petri dishes on a solid nutrient medium (Lactose TTX agar with tergitol 7) in appropriate dilutions to obtain single colonies. All nutrient medium and chemicals for them were from Federal Budget Institution of Science

State Research Center for Applied Biotechnology and Microbiology, Obolensk, Russia. One colony from the solid nutrient medium was transferred to 15 mL of LB medium and cultured overnight at 30 °C and 170 rpm. The resulting suspension in appropriate 10-fold

dilutions was plated on a solid medium to determine the exact number of viable cells. As a result, an *E. coli* suspension with a cell content of 2.5 × 10^9 CFU/mL was obtained. The resulting suspension was diluted to a certain cell concentration and 1 mL was added to 100 mL samples of "model" milk to a final concentration of 10^5 and 10^2 CFU/mL, respectively. Control samples containing 10^2 CFU/mL *E. coli* were kept at 30 °C for 24 h. The samples were sterilely collected after 6 and 24 h to determine the amount of *E. coli* using microbiological and molecular genetic methods. The real-time PCR was performed with a primer selected according to the literature to determine the total number of *E.coli* bacteria in the samples and to evaluate the relationship between the results of microbiological and molecular genetic methods.

Raw milk was standardized and pasteurized at (76 ± 2) °C under aseptic conditions to destroy coliform bacteria cells to obtain samples of drinkable milk. Control sample of drinkable milk was produced under aseptic conditions from the same normalized mixture with a fat mass fraction of 2.5%, cooked for 5 min, and, following this, cooled to (4 ± 2) °C. To study changes in the gas phase and sensor output data of milk samples during processing and correlation with microbiological and molecular genetic methods, an experiment was conducted with contamination of raw milk with a pre-determined chemical composition with *E. coli* bacteria using a cell suspension in the amount of 1.2 10^9 CFU/mL after pasteurization of the normalized mixture. The process of contamination of the pasteurized milk samples was identical to that for the reference samples. To assess and take into account the effect of milk sample temperature on sensor signals after measuring the gas phase in milk samples, the temperature was measured using a thermometer. The temperature of all milk samples varied from 24.6 to 25.0 °C. A total of four milk samples were analyzed in three time sections with an assessment of *E.coli* growth during cultivation in milk samples. To check the adequacy of the obtained models, three samples of raw milk containing pathogenic microorganisms (*Klebsiella*, *Staphylococcus aureus*) were additionally analyzed.

The gas phase of milk samples was studied using Diagnost-Bio-8 (OOO Sensino, Kursk, Russia) with a frontal input of the gas phase. An array was formed from sensors with polycomposite coatings of the following composition: (1) dicyclohexyl-18-crown-6/concentrate of micellar caseine; (2) dicyclohexyl-18-crown-6/chitosan; (3) Tween 40/Triton X-100; (4) choline + sorbitol + carbon nanotubes; (5) lanoline + silicone oxide; (6) lanoline + silicone oxide + choline + sorbitol; (7) dicyclohexyl-18-crown-6/Tritone X-100; (8) sorbitol + silicone oxide.

The preparation of sensors and their training on volatile vapors and water were carried out according to the method described in [27]. Then, the gas phase of the milk sample (V = 0.05 mL) was analyzed in the mode of 40 s sorption, 80 s desorption. The total time of one measurement is 120 s, and the analysis of each sample is carried out at least twice. Before the start of milk sample measurements and at the end of the working day, a distilled water sample is measured in a similar mode as an internal standard. Using the software of the device, the sensor signals were recorded for 40 s and 120 s of measurement. Then, the average sensor signals for milk and water samples were calculated, and the relative sensor signals ($\Delta F_{max,i}$ and $\Delta F_{120s,i}$) were calculated by dividing the average sensor signal values for milk samples by the average sensor signals in water vapor. The coefficient β_i, proposed earlier:

$$\beta_i = \frac{\Delta F_{max,i} - \Delta F_{120s,i}}{120 - \tau_{max,i}} \quad (1)$$

where $\Delta F_{max,i}$ and $\Delta F_{120s,i}$ are the maximum signal and signal at 120 s of measurement of i-th sensor, $\tau_{max,i}$—time of achievement of maximal signal by i-th sensor.

To assess the change in the composition of the gas phase of milk, the relative signal was calculated using Formula (2):

$$\Delta_i = \frac{\Delta F_{\max,i}(after\ processing) - \Delta F_{\max,i}(raw\ milk)}{\Delta F_{\max,i}(after\ processing)} \quad (2)$$

The resulting array of sensors was tested in vapors of volatile compounds present in the gas phase of milk, and the array was also tested in vapors of the gas phase of raw milk with different levels of QMAFAnM. Calibration of the array of sensors was performed using samples of the prepared standard contaminated with *E. coli* bacteria. The general plan of the study is presented in Figure 1.

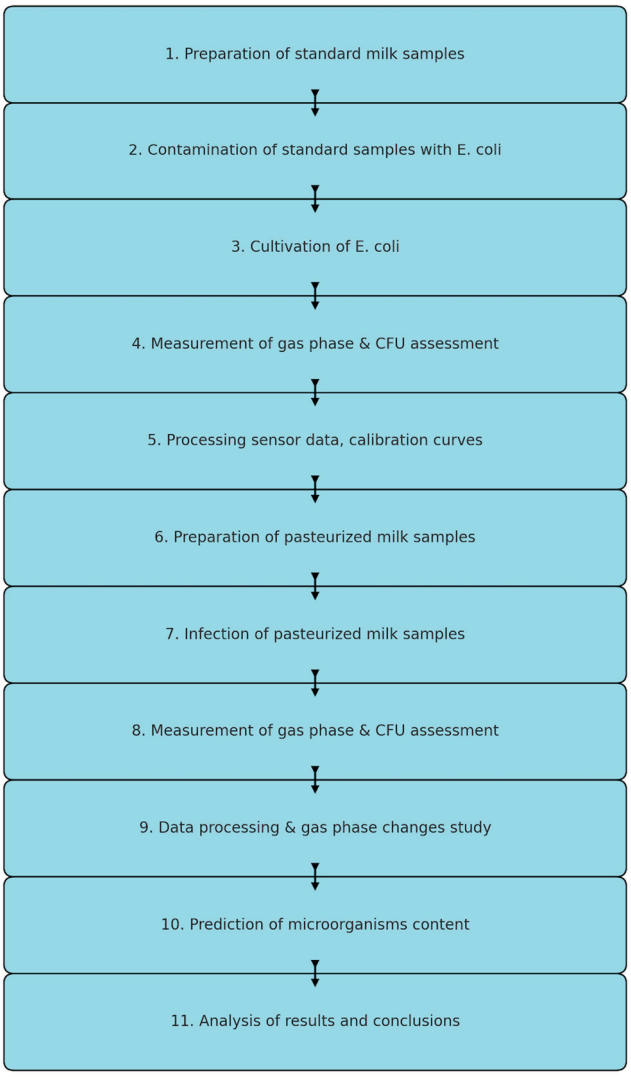

Figure 1. The flow chart of the experiment.

3. Results and Discussion

The results of determining the amount of *E. coli* in reference samples when kept at 30 °C for 6 and 24 h: the amount of *E. coli* increased to 2.5×10^5 CFU/mL in 6 h and to 1.2×10^8 CFU/mL in 24 h. Regularities of changes in sensor signals and calculated parameters depending on changes in the amount of *E.coli* in reference milk samples have been established. Calibration curves for predicting bacterial contamination of milk samples based on output data from sensors with polycomposite coatings have been constructed (Figure 2).

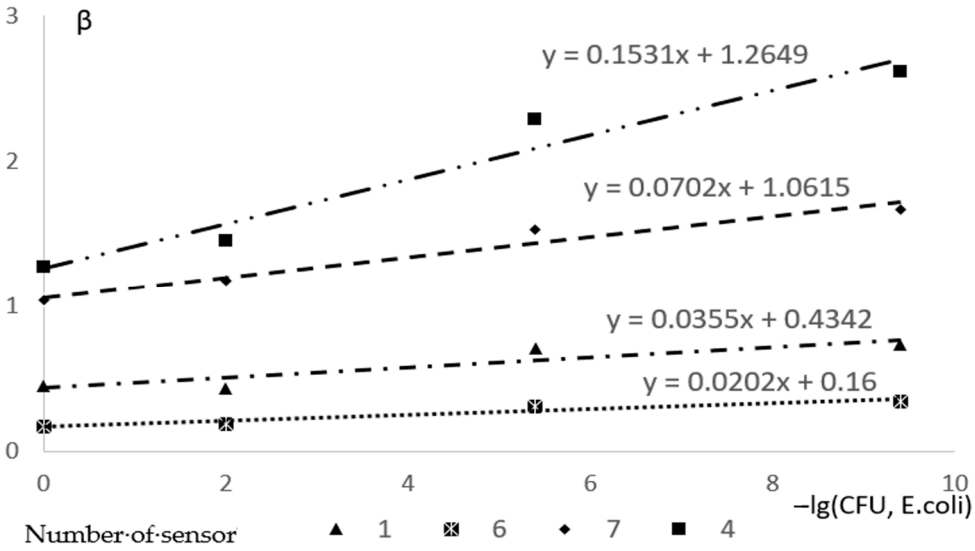

Figure 2. Calibration curves of the dependence of the coefficients β for sensors on lg(CFU) of QMAFAnM.

At each stage of the technological process, an analysis of the most significant physical and chemical indicators of all samples was carried out, as well as their compliance with the requirements of CU TR 033/2013 "On Safety of Milk and Dairy Products" for microbiological indicators (Table 2).

Table 2. Physicochemical parameters of milk samples during processing, including with *E. coli.*

Name of the Indicator	Sample						
	Reference No 1	**Raw Cow's Milk**	**Skim Milk**	**Normalized Mixture**	**Pasteurized Mixture**	**Drinking Milk (Experimental)**	**Drinking Milk (Control)**
Milk in dry matter, %	12.3–12.5	11.36 ± 0.30	9.71 ± 0.17	11.50 ± 0.38		11.58 ± 0.33	11.53 ± 0.41
Fat in dry matter, %	4.0–4.1	3.05 ± 0.05	0.05 ± 0.05	2.5 ± 0.05			
Total protein in dry matter, %	3.0–3.1	3.46 ± 0.1					
Lactose in dry matter, %	4.65–4.70	4.41 ± 0.36	Not determined	3.47 ± 0.15		3.10 ± 0.29	3.35 ± 0.20
Titratable acidity, °T	17 ± 0.5	18 ± 0.5	19 ± 0.5	17 ± 0.5	18 ± 0.5	18 ± 0.5	18 ± 0.5
Density, kg/m^3	1025 ± 0.5	1031 ± 0.5	1030 ± 0.5	1026 ± 0.5	1026 ± 0.5	1026 ± 0.5	1026 ± 0.5
QMAFAnM, CFU/mL	<10^3	5.8×10^6	Not determined		<10^3	Not determined	
Coliform bacteria, CFU/mL	0	Not determined			0	10^2	0

It has been shown that in the presence of contamination, a significant decrease in the amount of lactose in the milk sample occurs within two hours. At each stage of the technological process, the gas phase of the milk samples was analyzed using piezoelectric sensors with polycomposite coatings. The relative signal of each sensor (in relation to the raw milk sample—Δ, rel. units) was calculated, which, as a first approximation, can be used to estimate the change in the content of classes of volatile compounds, knowing the sensitivity of the sensors to these classes of compounds. The features of the change in the gas phase composition and the output data of the sensors at each stage of the technological process were established, which are consistent with the changes in the gas

phase determined by the gas chromatography method, including according to the literary data [28]. Compared with whole raw milk, during processing, depletion of the milk aroma is observed, primarily a decrease in the amount of volatile acids and an increase in the content of alcohols and ketones with a hydrocarbon radical C_5, while the strongest decrease in acids in the gas phase is observed after normalization and continues during pasteurization. At the same time, in the presence of contamination, a decrease in the content of short-chain alcohols and an increase in the content of propionic and butyric acids are observed compared to the original raw milk. Relative changes in sensor signals for different stages of the technological process are presented in Figure 3.

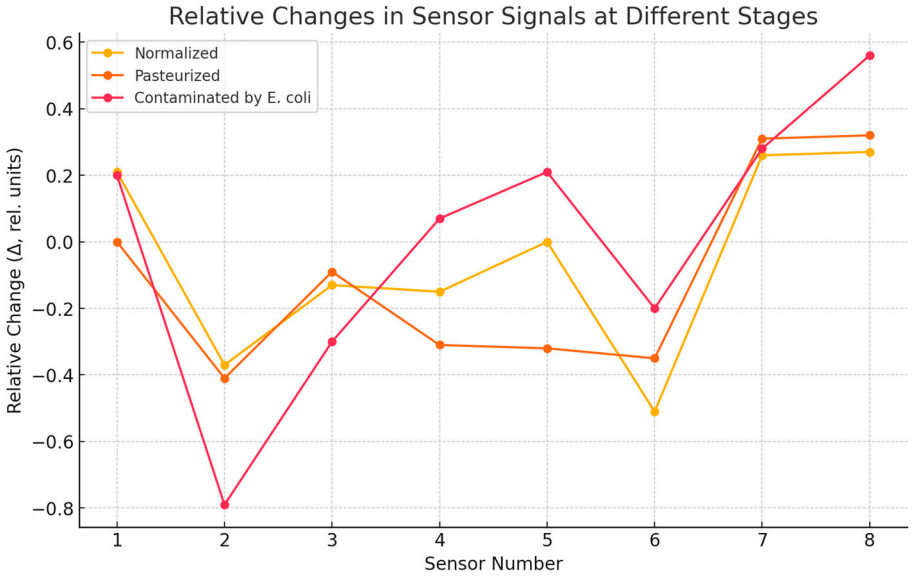

Figure 3. Relative changes in sensor signals (Δ_i, rel. units) for different stages in the technological process in relation to raw milk.

The results of QMAFAnM prediction using the calibration curves (Figure 2) are presented in Table 3.

Table 3. Results of QMAFAnM logarithm prediction (CFU/cm3) for experimental milk samples.

Sensor Number for Calibration	**Milk Samples**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
1	−0.67	0.41	−1.40	3.66	0.77	4.74	2.21
4	4.47	3.04	1.79	5.56	3.88	4.89	4.47
6	0.52	1.79	−2.02	3.06	1.15	3.06	3.06
7	−1.29	1.45	−3.48	4.01	1.81	2.36	0.90
CFU(*E. coli*) in cm^3	<10	10^2	<10	0	10^2	0	10^3
Notes	Presence of other pathogenic microorganisms			High level of QMAFAnM (>10^6) in raw milk	Artificial contaminated	Presence of mold (>600) in raw milk	Artificial contaminated

The estimate of the *E. coli* content in a milk sample is the arithmetic mean of the predicted values for each sensor. It has been noted that if a raw milk sample contains a large QMAFAnM (more than 10^6 CFU) or a high mold content (more than 600 CFU), the predicted values of the *E. coli* content may be overestimated even in the absence of live microorganisms in the milk. It was concluded that the obtained models can be used to

assess the presence and quantity of *E. coli* in milk at the pasteurization stage. This analysis method can be integrated into existing automation and control systems for technological equipment, such as a block module after a high-temperature short-time pasteurizer, which will allow for obtaining information about the presence or absence of *E. coli* in milk mixtures before subsequent stages of their processing and improve the accuracy of accounting for pasteurization modes. The main limitation of this approach now is the lack of large-scale testing in production, research on the adaptation of open-cell sensors to the conditions of a dairy production workshop (there should be no strong-smelling or volatile compounds in the measurement zone), and the need to adjust the automated process control system software (for example, ACS TP of the milk receiving, EKOMASH Enterprise, Russia; Software package MultiMilk expert, FoodSoft, Russia) to ensure the correct and dairy industry equipment continuity of service.

The obtained data indicate the limitations of the proposed approach—there is a fairly low specificity to the selected microorganism, an incompletely understood mechanism of the influence of variations in the physicochemical parameters of raw milk on the composition and content of volatile substances in the gas phase of milk, which are disadvantages when compared with other analysis methods, such as PCR and biosensors. However, at the same time, the proposed approach reduces time costs, and does not use sample preparation or additional reagents, which significantly reduces the cost of analysis and the impact on environmental harm and contributes to sustainable development.

To increase the reliability of the research, the following procedures can be used:

1. Use experimental design methods to assess the content of various microorganisms in milk.
2. Conduct repeated studies using raw milk with a wider variation in physicochemical parameters and in different seasons to account for the variation in the change in the native gas composition of milk depending on these factors.
3. Conduct additional studies to increase the accuracy of the analysis and take into account all possible factors that can affect the experiment.

The use of sensors is currently associated with the development of the Internet of Things and artificial intelligence. The development of artificial intelligence will contribute to the development of automation and control of the technological process; in addition, sensor-based systems will be able to learn additionally during operation in production, increasing the accuracy of analysis, automatically stop the technological process when regulatory indicators are violated, and, possibly in the future, also in a semi-automatic mode, give recommendations for adjusting the recipe depending on the raw materials used.

4. Conclusions

Thus, based on the results of checking the calibration graphs using milk samples as an example, limitations in the application of this approach were established. The proposed approaches to quantitative assessments of coliform bacteria in raw and pasteurized milk using gas-phase analysis with an array of sensors make it possible to significantly reduce the analysis time to 2–3 h (including the sample collection and data processing) and thereby intensify the production of safe dairy products. This approach can be integrated into the process flow if milk samples are taken at production control points with subsequent analysis in the laboratory. In the future, when studying the adaptation of sensors to the dairy production shop, this type of analysis can be automated and integrated into the process line. The analysis time for a sample of chilled milk does not exceed 3 min. It is also planned to increase the selectivity of the sensors to volatile compounds released by pathogenic microorganisms against the background of the natural aroma of milk.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author (ethical reasons from milk manufacturer used in this investigation).

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

OJSC	Open joint stock company
DNA	Deoxyribonucleic acid
PJSC	Public joint stock company
PCR	Polymerase chain reaction
CFU	Colony-forming unit

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