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Bacteriological quality and antimicrobial resistance of *Staphylococcus* spp. and *Escherichia coli* isolated from organic and conventional fresh cheese

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Abstract

One of the appeals of the rising organic market is the guarantee of offering healthier foods with less impact on the environment, from a more sustainable production method. Dairy market is one of the most popular in the sector but studies on organic dairy products safety are still scarce. The objective of this study was to evaluate the microbiological quality of organic and conventional Minas Frescal cheese samples, and the antimicrobial sensitivity of isolated strains of *Escherichia coli* and coagulase-positive *Staphylococcus* to different antimicrobials. *Listeria* spp. and *Salmonella* spp. was not detected in any of the samples analyzed. Regarding coagulase-positive *Staphylococcus*, 70% showed higher counts than that is established by Brazilian legislation, but with no significant difference between the systems. In the determination of the Most Probable Number of *E. coli* was observed significant difference between the systems, with a higher contamination index in cheeses derived from the organic system. All strains isolated showed 100% resistance to β-lactams and both in the conventional and organic systems were observed multiple resistance characteristics. Considering the similarity of the results obtained, it is necessary to analyze other parameters such as production system, herd health and good manufacturing practices to compare deeply both systems.

Keywords: organic dairy; foodborne pathogens; antimicrobials; multiple resistance.

Practical Application: In view of the widespread consumption of cheeses and the rise of the organic market in the world, it is important to guide consumers about the quality and safety of these products considered superior to conventional products.

1 Introduction

The trend towards healthy eating has had a major impact on food production, generally linked to concerns about animal welfare and environmental issues. This aspect is considered to increase the demand for organic foods, seen as healthier and with less impact on the environment, coming from a more sustainable production method than the conventional one (de-Magistris & Gracia, 2016).

In the organic market, milk and dairy products are considered to represent the second largest share, behind only of the largest of them, the fruit and vegetable sector (Research Institute of Organic Agriculture, 2018). Organic-certified foods occupy the "premium" food market segment, targeting consumers willing to pay higher prices for selected products, considered to be of better quality and safety (Heckman, 2019).

Significantly higher prices are justified not only by the market rising but also by the higher production cost when compared to the conventional system (Badruddoza et al., 2022). Although the requirements for the certification of an organic product vary according to the regulatory agency of each country, the requirements commonly demanded are expensive and require specialized labor (Rotz et al., 2007).

Among the cheeses widely spread in Brazil, Minas Frescal cheese stands out as the third most consumed. As the name classifies, it is a fresh, curdled, unripened, of very high moisture and semi-fat cheese (Brasil, 2004; Empresa Brasileira de Pesquisa Agropecuária, 2019). Such features provide a favorable growth environment for contaminants, mainly bacterial pathogens (Delorme et al., 2020).

The main pathogens isolated from cheeses are *Escherichia coli*, *Staphylococcus* spp., *Salmonella* spp. and *Listeria monocytogenes*, commonly related to foodborne disease outbreaks worldwide, considered to be one of the main threats to public health (Chavez-Martinez et al., 2019; Lee & Yoon, 2021). Linked to this, the emergence of multidrug-resistant strains caused by the indiscriminate use of antimicrobials increases the difficulty of clinical treatment of these manifestations (Ge et al., 2022).

In conventional animal production, antibiotics are used for therapeutic or prophylactic purposes for diseases that impact animal health and production volume, but when misused, they can be harmful for both the environment and consumers health (Jayarao et al., 2019; Sharma et al., 2018). In organic production, the use of antimicrobials is prohibited, except when the use of

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permitted alternative medications has no effect and the animal is under suffering or at risk of death (Brasil, 2011).

The presence of lactic acid bacteria in cheeses is favorable in terms of sensory characteristics, as it imparts flavor, aroma and texture, and in terms of microbiological quality, considering its ability to produce antimicrobial substances, known as bacteriocins (Antônio & Borelli, 2020). Its antagonistic action can be observed against important pathogens, such as *Listeria monocytogenes* and *Staphylococcus aureus* (Darbandi et al., 2022).

Given the significant increase of organic cheese consumption in the world and the lack of scientific evidence about the superior quality of the dairy products mentioned, the study aimed to evaluate the bacteriological quality of organic and conventional fresh cheeses, and to estimate the resistance of coagulase-positive *Staphylococcus* and *Escherichia coli* to different antimicrobials.

2 Material and methods

2.1 Sample collection and preparing

Thirty samples of Minas Frescal cheese were collected in supermarkets and organic food stores in the state of Rio de Janeiro. Fifteen samples originated from each system were from 5 different brands, which brand collected from three distinct stores and manufacturing dates, from May to July 2021. The packaging of the samples was carried out in isothermal boxes with reusable ice, maintaining the appropriate temperature conditions (from 6 °C to 8 °C) during transport to the Microbiological Control of Animal Origin Products Laboratory, of Universidade Federal Fluminense. The samples were separated and identified according to classification (conventional and organic) and prepared according to the bacteriological analyzes to be performed.

Twenty-five grams of each sample were aseptically homogenized in 225 mL of 0.1% peptone saline solution, obtaining the first dilution (10^{-1}). Subsequent dilutions up to 10^{-7} were derived from this initial dilution. For the detection of *Salmonella* spp., 25 g of the sample was aseptically homogenized in 225 mL of 1.0% buffered peptone water, while for *Listeria* spp., 25 g aliquots were diluted in 225mL of Half Fraser Broth (Himedia, Mumbai, India). The samples were homogenized using a Stomacher® homogenizer (Seward, Worthing, UK) at medium speed for 60 seconds.

2.2 Microbiological evaluation

The determination of the Most Probable Number (MPN) of total coliforms, thermotolerant coliforms and *E. coli* was performed according to Merck methodology (Merck, 2000), using Fluorocult Lauryl Sulfate Broth (Merck, Germany), confirmed by Kovacs test. All confirmed strains were subcultured into Brain Heart Infusion (BHI) broth (Himedia, Mumbai, India), and then inoculated into Eosin Methylene Blue agar plates (BD, Heidelberg, Germany) for isolation of strains.

Coagulase-positive *Staphylococcus* counting was performed as described in the Compendium of Methods for the Microbiological Examination of Foods (Bennett et al., 2015), by spread plate inoculation in Baird Parker agar (Kasvi, Paraná, Brazil) plates. Five suspected colonies were selected from each plate, confirmed by coagulase and catalase tests and morphotintorial characterization by smear and Gram staining.

The lactic acid bacteria (LAB) count was performed according to methodology described by Frank & Yousef (2004) in the Standard Methods for the Examination of Dairy Products from inoculation by overlay on Man, Rogosa and Sharpe agar (Neogen, Michigan, USA).

The detection of *Listeria* spp. was performed by the plating method described by Ryser & Donnelly (2015) in the Compendium of Methods for the Microbiological Examination of Foods using Half-Fraser broth in the pre-enrichment step, followed by enrichment in Fraser broth (Himedia, Mumbai, India) and subsequent plating on Modified Oxford agar (Himedia, Mumbai, India) and *Listeria* acc. Ottaviani & Agosti (Kasvi, Paraná, Brazil).

Salmonella spp. detection was performed by the methodology described in the Compendium of Methods for the Microbiological Examination of Foods (Cox et al., 2015), starting with pre-enrichment in 1% buffered peptone saline solution, followed by selective enrichment in Mossel (Oxoid, Hants, UK) and Rappaport Vassiliadis broth (Oxoid, Hants, UK), with subsequent selective plating using Brilliant Green, Xylose Lysine Deoxycholate and *Salmonella* spp. Differential agar (Himedia, Mumbai, India).

All isolated strains of *E. coli* and *Staphylococcus* spp. were transferred to BHI broth and kept in a refrigerator at an average temperature of 4 °C for antimicrobial susceptibility test.

2.3 Antimicrobial susceptibility test

The test was performed according to the recommendations of the Clinical & Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2021), by the agar disk-diffusion test, also known as Kirby-Bauer Method (Bauer et al., 1966) and the antimicrobials were chosen considering the most relevant antimicrobials in terms of frequency of use and impacts on public health.

Strains were standardized to category one on the McFarland Standards (Probac, Brazil), an universally established scale to standardize the approximate number of bacteria in a liquid suspension, which, in category one represents the concentration of 3 x 10^8 bacteria/mL, seeded on plates containing Müeller Hinton Agar. The antimicrobial discs arranged in modules, of 6 antimicrobial discs per module (Polisensidisc 24, DME), were superimposed on the plates. For both microorganisms, the following antimicrobials were used: Aztreonam (ATM 30 µg), Cefoxitin (CFO 30µg), Chloramphenicol (CLO 30 µg), Gentamicin (GEN 10 µg) and Tetracycline (TET 30 µg). Specifically for *E. coli*, were also used: Amikacin (AMI 30 µg), Ampicillin (AMP 10 µg), Cefazolin (CFZ 10 µg), Cefotaxime (CTX 30 µg), Ceftazidime (CAZ 30 µg), Sulfamethoxazole + Trimethoprim (SUT 25 µg) and Ceftriaxone (CRO 30 µg). For *Staphylococcus* spp., Clindamycin (CLI 02 µg), Erythromycin (ERI 15 µg), Oxacycline (OXA 01 µg), Penicillin G (PEN 10 µg), Teicoplamine (TEC 30 µg) and Vancomycin (VAN 30 µg) were also tested.

After incubation at 35-37 °C for 24 hours, the test was interpreted using standard measures of inhibition halos for each antimicrobial used according to the table established in

the CLSI (Clinical and Laboratory Standards Institute, 2021) using a halometer to classify the strains as resistant, intermediate or sensitive.

2.4 Statistical analysis

For the statistical analysis of the data, non-parametric tests were applied. Friedman's test was applied to compare the values of the two groups, appropriate or inappropriate for consumption, while Pearson's linear correlation coefficient was used to evaluate potential associations between the variables and between conventional and organic matrices, using also Mann-Whitney Test to compare both systems regarding quantitative variables, considering the level of global significance of 5% ($p \leq 0.05$). Data were processed using the IBM "Statistical Package for the Social Sciences" (SPSS), version 18.0.

3 Results and discussion

The results, described in Table 1, were compared with the microbiological standards for foods pre-established in Brazil by IN n°60 (Normative Instruction n° 60, of December 23, 2019, of Anvisa), based on the category of cheeses with moisture above 46%, classifying the samples as appropriate or inappropriate for consumption (Brasil, 2019).

Coagulase-positive *Staphylococcus* counts ranged from 0 to 7.4 log CFU/g in conventional cheeses and from 0 to 7.9 log CFU/g in organic cheeses. Of the 30 samples analyzed, 21 samples (70%), being 13 (43.4%) conventional and 8 (26.6%) organic, presented counts higher than the recommended in Brazilian legislation, of 3 log CFU/g, as described in Figure 1. Therefore, they were unfit for consumption (Brasil, 2019), but with no statistical difference between the production systems regarding consumption suitability ($p > 0.05$).

Mullen et al. (2013) stated that despite differences in herd management, the prevalence of *Staphylococcus* spp. in the herd was remarkably similar between the organic and conventional dairies surveyed, and that conventional and organic dairy farmers face similar challenges in mastitis management and the quality of milk produced. Similarities in the systems were also reported by Malissiova et al. (2017), in a study about differences in the microbiological profile of milk from sheep and goats between conventional and organic farming systems in Greece.

In conventional cheese, the MPN of *Escherichia coli* ranged from 0 to 1.5 log NMP/g, and in none of the 15 samples analyzed was a count higher than that determined in the legislation of 3.0 log MPN/g. In the organic cheeses, the MPN ranged from 0 to 11.9 log MPN/g and two of the 15 samples were inappropriate for consumption. The significant difference in total coliforms and *E. coli* count was observed between the systems ($p = 0.002$ and $p = 0.042$, respectively), with a higher contamination index in cheeses derived from the organic system. Higher absolute levels of *E. coli* contamination in organic cheeses were also reported by

Table 1. Mean bacterial counts of organic and conventional Minas Fresh cheese.

	Coagulase-positive *Staphylococcus* (log CFU/g)	Total Coliforms (log MPN/g)	*E. coli* (log MPN/g)	LAB (log CFU/g)	*Salmonella* spp.	*Listeria* spp.
Conventional	4.7 ± 0.56[a]	2.8 ± 0.45[a]	0.1 ± 0.1[a]	7.4 ± 0.56[a]	ND	ND
Organic	3.4 ± 0.89[a]	4.9 ± 0.39[b]	2.37 ± 0.98[b]	8.4 ± 0.36[a]	ND	ND
p-value	0.513	0.002	0.042	0.091	-	-

CFU/g = Colony Forming Unit per gram. MPN/g = Most Probable Number per gram. LAB = Lactic Acid Bacteria. Results are presented as mean ± standard deviation of duplicate measurements. a, b = compare the significance value of p< 0.05. ND: below the limit of detection.

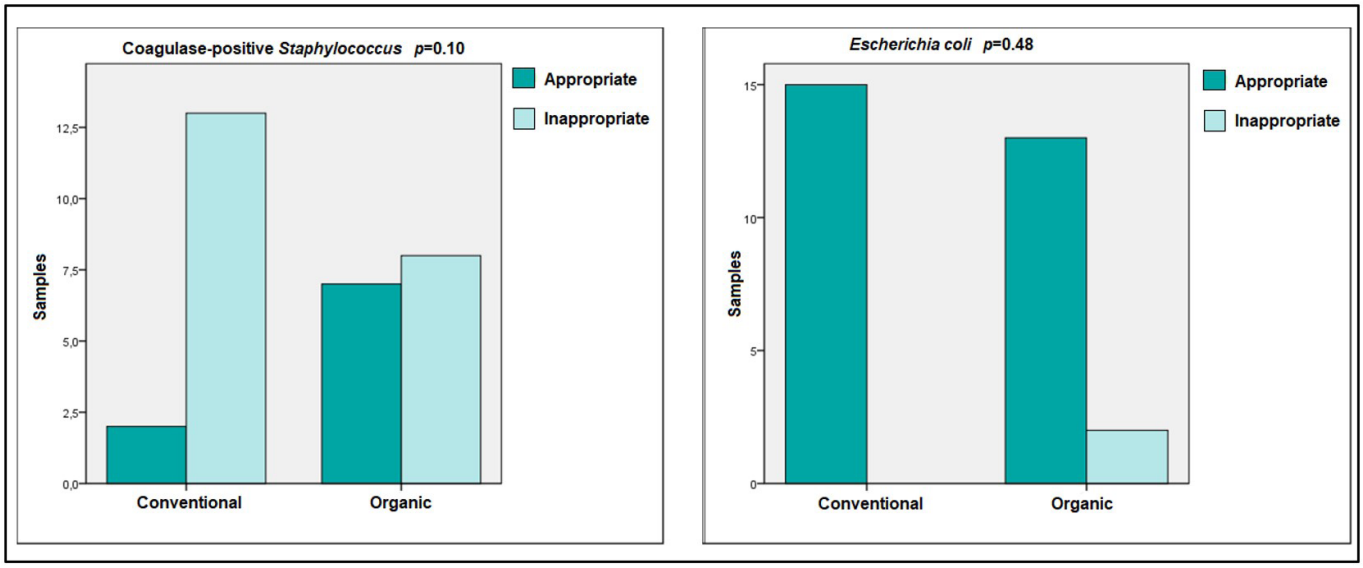

Figure 1. Suitability of conventional and organic Minas Frescal Cheese samples according to IN 60 (Brasil, 2019) for coagulase-positive *Staphylococcus* and *Escherichia coli* counts.

Kukułowicz (2018) and Wanniatie et al. (2019) in comparative studies about different organic dairy products and organic goat milk, respectively. On the other hand, no statistical difference was observed in both related studies.

The presence of *E. coli* in dairy products, an indicator of fecal contamination, is mainly associated with the use of raw or improperly pasteurized milk and poor hygienic-sanitary practices during processing (Hammad et al., 2022), determinant factors in both production systems.

Regardless of the cheeses origin, *Salmonella* spp. and *Listeria* spp. were not detected in the analyzed samples, in accordance with the provisions of national legislation (Brasil, 2019) and with what was reported by Messias et al. (2022), in an analysis of different typical Brazilian cheeses and by Kukułowicz (2018), who, when comparing different dairy products of conventional and organic origin, did not detect the presence of *Salmonella* spp. in any of the samples analyzed. Sosnowski & Osek (2021) observed that despite reports by several authors about the presence of *Listeria* spp. in different organic and conventional matrices there is no significant difference between the production systems on the presence of *Listeria* spp.

Van Loo et al. (2012) considered that organic production poses a risk in relation to microbiological safety arising from system particularities such as restriction of the use of antimicrobials and antiparasitics and outdoor breeding, favoring the spread of zoonosis due to greater exposure of the animal. Disagreeing, Galdino et al. (2012) reported that such obstacles can be mitigated by the adoption of strict hygiene practices and the application of complementary therapy, such as homeopathy, acupuncture and herbal medicine. Furthermore, Schwendel et al. (2015) reported that numerous factors must be considered when evaluating the differences between organic and conventional dairy, from minimal factors such as animal breed and diet composition to production and post-processing handling.

The lactic acid bacteria count ranged from 4.9 to 9.0 log CFU/g in conventional cheeses and from 5.1 to 10.6 log CFU/g in organic cheeses. Result that, when analyzed using Pearson's correlation method with the count of *E. coli* and coagulase-positive *Staphylococcus*, did not establish a correlation ($r = +0.28$; $p = 0.12$ for *E. coli* and $r = -0.24$ and $p = 0.19$ for coagulase-positive *Staphylococcus)*, indicating that the presence of LAB had no influence over the presence of pathogens. Differently from the reported by Pato et al. (2022) when evaluating the antimicrobial activity of lactic acid bacteria and bacteriocins isolated from Dadih (traditional fermented milk from Indonesia) on *Staphylococcus aureus* strains, in which all 12 isolated strains were able to suppress the growth of *S. aureus*. Santos et al. (2019), in a study on the probiotic and molecular characterization of LAB isolated from cheese, noted that probiotic characterization is extremely necessary since not all LAB have antimicrobial characteristics. In the aforementioned study, of the 11 bacteria isolated, only two showed probiotic potential. According to the parameters published by the National Health Surveillance Agency (Brasil, 1999), to classify a product as a probiotic the minimum concentration of viable probiotic microorganisms need to be achieved, of 8 to 9 log CFU/mL, persistent till the end of the shelf life.

The characterization of the antimicrobial resistance of coagulase-positive *Staphylococcus* strains to different antimicrobials is described in Table 2. All strains isolated from conventional and organic cheeses showed multi-resistance and there was no statistical difference between the systems.

The class of antimicrobials that showed the lowest efficacy on isolated coagulase-positive *Staphylococcus* strains was the β-lactams such as oxacyclin, penicillin and aztreonam. Similar results were reported by Abreu et al. (2021) when analyzing the antimicrobial resistance of strains of *Staphylococcus* spp. isolated from conventional and organic Minas Frescal cheese, which consider that there are several factors that influence the sensitivity of microorganisms isolated in dairy products, such as contamination by handlers during processing, and that the ineffectiveness of β-lactams is justified by the popularity and extensive use of the class in the country, ineffectiveness also reported by Keyvan et al. (2020).

Of the six strains of *E. coli* isolated in the research, five belonged to organic cheeses while only one strain was isolated from conventional cheeses. The strain resistance profile is described in Table 3.

Table 2. Susceptibility of coagulase-positive *Staphylococcus* to antimicrobials.

Antimicrobial	Conventional (n = 13)			Organic (n = 8)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Clindamycin	3 (23.1%)	6 (46.1%)	4 (30.8%)	2 (25%)	0 (0%)	6 (75%)
Erythromycin	6 (46.1%)	4 (30.8%)	3 (23.1%)	0 (0%)	3 (37.5%)	5 (62.5%)
Oxacyclin	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	8 (100%)
Penicillin G	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	8 (100%)
Teicoplamine*	12 (92.3%)	0 (0%)	1 (7.7%)	4 (50%)	0 (0%)	4 (50%)
Vancomycin*	12 (92.3%)	0 (0%)	1 (7.7%)	4 (50%)	0 (0%)	4 (50%)
Aztreonam	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	8 (100%)
Cefoxitin	0 (0%)	0 (0%)	13 (100%)	0 (0%)	0 (0%)	8 (100%)
Chloramphenicol	13 (100%)	0 (0%)	0 (0%)	7 (87.5%)	0 (0%)	1 (12.5%)
Gentamicin	10 (76.9%)	1 (7.7%)	2 (15.4%)	6 (75%)	1 (12.5%)	1 (12.5%)
Tetracyclin	11 (84.6%)	1 (7.7%)	1 (7.7%)	7 (87.5%)	1 (12.5%)	0 (0%)

*p value ≤ 0.05.

Table 3. Susceptibility of *E. coli* to antimicrobials.

Antimicrobial	Conventional (n = 1)			Organic (n = 5)		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Amikacin	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1 (20%)	4 (80%)
Ampicillin	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	5 (100%)
Cefazolin	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	5 (100%)
Cefotaxime	0 (0%)	0 (0%)	1 (100%)	1 (20%)	0 (0%)	4 (80%)
Ceftazidime	0 (0%)	0 (0%)	1 (100%)	2 (40%)	1 (20%)	2 (40%)
Sulfa + Trimethoprim	0 (0%)	0 (0%)	1 (100%)	2 (40%)	0 (0%)	3 (60%)
Aztreonam	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	5 (100%)
Cefoxitin	0 (0%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	5 (100%)
Ceftriaxone	0 (0%)	0 (0%)	1 (100%)	1 (20%)	3 (60%)	1 (20%)
Chloramphenicol	1 (100%)	0 (0%)	0 (0%)		0 (0%)	0 (0%)
Gentamicin	1 (100%)	0 (0%)	0 (0%)		1 (20%)	0 (0%)
Tetracycline	1 (100%)	0 (0%)	0 (0%)		0 (0%)	0 (0%)

All strains isolated showed multidrug resistance, with 100% resistance to ampicillin, cefazolin, aztreonam and cefoxitin in both production systems. The disparity in the number of strains isolated from conventional and organic cheeses made statistical analysis of the data difficult when comparing the systems.

The predominance of *E. coli* resistance to ampicillin in milk and dairy products was also reported by Gundogan & Avci (2014), who justified this incidence by the routine use of β-lactams in the treatment of *E. coli* infections in both humans and animals and by the strains ability to produce extended-spectrum β-lactamases, enzymes capable of inactivating antimicrobials of the class.

Almeida et al. (2021), when evaluating the presence and antimicrobial resistance of Enterobacteriaceae in calves, cows and the milking environment, observed a high rate of ampicillin resistance and multidrug resistance in all isolates. Characteristics that, according to Poirel et al. (2018), are extremely important from a One Health perspective due to the worldwide spread of *E. coli*, commonly associated with food, in which resistance to multiple classes of antimicrobials leads to difficult-to-treat infections.

Results contrasting with those found by Sato et al. (2005) when analyzing isolates from cows and calves from conventional and organic systems, which reported a low rate of resistance to ampicillin (19.5% in conventional and 10.4% in organic) and cefoxitin (1.4% in conventional and 0.4% in organic) and 100% amikacin sensitivity. Such differences between studies that are just over a decade apart reinforce the advance of antimicrobial resistance, currently considered a pandemic, with projection of causing about 10 million deaths by 2050 (Singhal, 2022).

4 Conclusions

The significant difference between the production systems regarding *E. coli* count is more commonly related to poor hygienic-sanitary conditions than to the type of production system. All other similarities found in this research reinforce the need to analyze other parameters that influence food safety such as herd health and good manufacturing practices, in order to justify the appealing of paying more for better quality food.

Regardless of the production system, the use of antimicrobials must be reconsidered in order to stop growing antimicrobial resistance. So that all types of consumers have access to a safe food, mitigating the risks of serious and/or difficult-to-treat foodborne diseases.

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