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Shiga Toxin-Producing *Escherichia coli* (STEC) O157:H7 in Beef

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Abstract

This study aimed to assess the hygienic-sanitary quality of raw beef sold in supermarkets and street markets in the Recôncavo region of Bahia, Brazil. Between May and July 2023, twenty-four samples were collected from six municipalities. The analyses included counts of mesophilic and psychrotrophic aerobic microorganisms, total coliforms, *Escherichia coli*, and the detection of *Salmonella* spp. Isolates of *E. coli* were subjected to genotypic characterization by Polymerase Chain Reaction (PCR) to identify virulence genes, focusing on the Shiga toxin gene (*stx*), indicative of enterohemorrhagic *E. coli* (EHEC), and the ST-I toxin gene (*stI*), associated with enterotoxigenic *E. coli* (ETEC). Results revealed that 75% of the samples did not comply with the microbiological standards established by current legislation for one or more tested microorganisms. Compliance was observed only in 25% of the establishments, exclusively in supermarkets. The *stI* gene was not detected in any isolate, while the *stx* gene was identified in two samples collected from street markets in the municipalities of Cachoeira and Santo Amaro. These findings indicate that a significant portion of beef marketed in the region presents unsatisfactory microbiological quality, posing potential risks to consumer health.

Keywords

Public Health, Food Safety, Genetic Characterization, Microbiology

1. Introduction

As the world's second-largest producer and exporter of beef, Brazil currently commands a significant share of the global market [1]. The industry's considerable modernization, which has increased productivity and raised product quality, is what has led to this leadership. Beyond its economic significance, the production and

trade of beef are essential to maintaining food security on a national and global scale [2].

Beef, which comes in a variety of cuts and presentation styles, is widely consumed and is acknowledged as one of the animal-based proteins with the highest biological value and greatest adaptability [3]. Animal muscle tissue is regarded as sterile under ideal circumstances [4]. Nonetheless, its inherent qualities—such as a pH that is nearly neutral, high water activity, and a rich nutritional makeup—provide an environment that is favorable for the development and proliferation of microorganisms [5].

Furthermore, meat can be exposed to different contaminants throughout the production chain—from slaughter, skinning, and evisceration, until it reaches the points of sale. Along this path, irregularities related to the storage, handling, and display of the product are common, often without due control and inspection by the competent bodies. These factors increase the risk of contamination, compromising the safety and quality of the meat [6] [7].

In this context, controlling the presence and multiplication of microorganisms becomes an essential criterion to ensure the sanitary and commercial quality of the product, since high levels of contamination not only favor its deterioration, but also represent serious risks to consumer health [8].

With the growth of national and international trade in animal products, the global spread of foodborne pathogens has become a growing concern. It has been demonstrated that food of animal origin can act as vectors for pathogenic microorganisms such as *Salmonella* spp., Shiga toxin-producing *Escherichia coli* (STEC), and *Listeria monocytogenes*. Beef, in particular, is occasionally consumed raw or undercooked, which increases the risk of ingesting viable pathogens, either directly or through cross-contamination. In this scenario, *Salmonella* spp., *L. monocytogenes*, STEC, and *Staphylococcus* spp., especially *Staphylococcus aureus*, stand out among the main agents causing foodborne diseases, being recognized as serious public health problems at a global level [9]-[11].

According to the World Trade Organization (WTO), more than 3 million children under the age of five die annually from foodborne diseases, often associated with severe diarrhea [12] [13]. In Brazil, microbial contamination is identified as the main cause of food poisoning outbreaks. Among the most frequently identified microorganisms are strains of *Salmonella*, *Escherichia coli* O157, *Listeria monocytogenes*, and *Shigella*, which may be present in meat products and pose serious risks to human health [13] [14]. It is important to note that meat can harbor multiple pathogens simultaneously, further aggravating the risks to public health. In some countries, such as China, the presence of these bacteria in meat products is strictly prohibited by health regulations.

Given this situation, the current study set out to assess the microbiological quality and carry out the genotypic characterization of *Escherichia coli* isolates from raw beef that was offered for sale in the Recôncavo Baiano region's supermarkets and street markets. The goal is to provide pertinent information to support food

safety initiatives and safeguard the health of consumers in the municipalities under study.

2. Material and Methods

2.1. Collection, Sample Preparation, and Microbiological Analysis

The study comprised visits to the twenty municipalities that make up the Recôncavo region of Bahia, with the objective of mapping beef marketing units. Among these, six municipalities (Santo Antônio de Jesus, Cruz das Almas, Muritiba, São Félix, Cachoeira, and Santo Amaro) were selected, as they are considered the most representative in terms of beef production, given that the region is predominantly characterized by poultry farming. In these localities, twelve beef marketing establishments, including supermarkets and street markets, were randomly selected in order to minimize potential selection bias and to ensure greater representativeness of the regional marketing profile.

Two visits were conducted in each municipality, and during each visit, two samples of raw beef (beef chuck), weighing 200 g each, were collected, totaling 24 samples between May and July 2023. It should be noted that the number of collections could not be expanded due to safety concerns, since a considerable portion of the marketed meat showed evidence of originating from clandestine slaughter, which constitutes a sanitary crime and requires caution during the execution of the study.

In order to preserve their original characteristics, the beef samples were aseptically collected using disposable gloves and previously sterilized instruments, and individually packed in sterile bags identified with code, location, date, and time of collection. Immediately afterwards, they were kept refrigerated in insulated boxes with recyclable ice packs, ensuring a controlled temperature between 2˚C and 8˚C during transport, in accordance with international recommendations for the transport of samples intended for microbiological analysis of food [15] [16].

The maximum time between collection and arrival at the laboratory did not exceed 24 hours. Upon arrival at the Animal Microbiology and Parasitology Laboratory of the Federal University of Recôncavo da Bahia (UFRB), Cruz das Almas campus, the samples were immediately inspected for package integrity and recorded in a traceability spreadsheet. They were then stored under refrigeration at 4˚C ± 1˚C and processed within 24 hours after reception, avoiding freezing in order not to compromise microbial viability, as recommended by Brazilian legislation [17] [18]. Handling was carried out in a biological safety cabinet, following biosafety protocols and good laboratory practices.

For microbiological analyses, regarding the presence of mesophilic and psychrotrophic aerobic microorganisms, total coliforms, and *Escherichia coli*, the samples were initially prepared aseptically and subjected to the serial dilution process until the third dilution (10^3) was reached, with the aim of reducing the contamination load per unit volume, making it possible to count the colonies in Petri dishes.

Then, 1 mL (1000 µL) aliquots of each dilution were added to sterile Petri dishes, and then the specific culture media for each microorganism were poured, using the "Pour Plate" deep plating technique.

For the analysis of total coliforms and *E. coli*, the Chromocult® Coliform Agar culture medium was used. As for the mesophilic and psychrotrophic aerobic microorganisms, the Standard Counting Agar (PCA) medium was used.

In the present study, additional biochemical tests or serotyping were not required, since the Chromocult® chromogenic medium was used. This medium is selective and specific for the identification of *Escherichia coli* and total coliforms, allowing direct colony differentiation through the expression of characteristic enzymes. It ensures high sensitivity and specificity for primary diagnosis, as recommended by international food microbiology protocols.

After inoculation of the samples, the plates were incubated inverted in an incubator at 37˚C for 24 hours to determine total coliforms and *E. coli*, and for 48 hours to determine mesophilic aerobes. The psychrotrophic bacteria were incubated in a refrigerator at 7˚C for ten days. After the incubation period, colony counts were performed using the standard plate count technique to express the results in Colony Forming Units per gram of sample (CFU/g). Finally, the data were subjected to statistical analysis using the Statistical Analysis System (SAS), and the results were expressed in log CFU/g.

Colonies suggestive of *E. coli* were preserved in Eppendorf microtubes containing Brain Heart Infusion (BHI) broth with 20% glycerol and kept at −18˚C for subsequent DNA extraction and Polymerase Chain Reaction (PCR) analysis.

The analysis for the research of *Salmonella* spp. was performed using the Compact Dry SL® chromogenic enzymatic method (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), performed according to the manufacturer's recommendations. After the incubation period, the results were expressed qualitatively, regarding the presence or absence of the agent.

2.2. DNA Extraction and PCR

For DNA extraction, the samples were initially recovered in BHI broth at 37˚C for 24 hours in a microbiological incubator, and then sent to the Genetics Laboratory-LIPAGE of the Federal University of Recôncavo of Bahia (UFRB), Santo Antônio de Jesus campus.

The Eppendorf microtubes containing 1 mL of the bacterial culture suspension were centrifuged at 13,200 rpm for 5 min. The supernatant was discarded, and then 800 µL of sterile milliQ water was added. After homogenization, the samples were again subjected to the centrifugation process under the same conditions described previously. Finally, the supernatant was discarded and 80 µL of sterile milliQ water was added. Then, the samples were subjected to a temperature of 96˚C for 10 min in a water bath. The supernatant was again discarded, and the samples were kept at −20˚C until the time of analysis.

The extracted DNA was subjected to PCR to identify the virulence genes *stI* and

stx, using the following reagents and volumes: 2.5 µL of PCR buffer; 0.5 µL of dNTP mix; 0.75 µL of $MgCl_2$; 0.2 µL of Taq DNA polymerase; 1.0 µL of primers 1 and 2; 3.0 µL of template DNA, with the final volume adjusted to 25 µL with sterile milliQ water.

The amplification reactions were performed in an Amplitherm® TX96 Plus thermocycler, according to the conditions, primer sequence, and fragment size (bp) described in Table 1.

The amplification products, together with SYBR Green Life Technologies®, were subjected to electrophoresis in a 2% agarose gel in a horizontal system, with the negative control of both genes studied. The run was performed with a GSR® 200STD digital source, in 1 hour, at 100 Volts and 40 mA. The products were then observed in a Loccus L-PIX ultraviolet photodocumenter.

Table 1. Primer sequence, amplified fragment size, and conditions used in PCR for detection of the virulence-associated gene.

Gene/serotype	Primer sequence 5'-3'	(bp)	PCR conditions
stx/EHEC	TTT ACG ATA GAC TTC TCG AC CAC ATA TAA ATT ATT TCG CTC	227	5 min 94˚C/35 cycles of 1 min 94˚C, 3 min 48˚C, and 4 min 72˚C/10 min 72˚C
stI/ETEC	TTA ATA GCA CCC GGT ACA AGC AGG CTT GAC TCT TCA AAA GAG AAA ATT AC	147	5 min 94˚C/30 cycles of 1 min 94˚C, 1 min 55˚C, and 1 min 72˚C/10 min 72˚C

3. Results and Discussion

E. coli O157, *Shigella*, and *Salmonella* are common foodborne pathogens that can cause a variety of illnesses, ranging from fever and diarrhea to abdominal pain and even death. Consequently, rapid, sensitive, and reliable detection of these pathogens is essential to mitigate the associated risks. In the field of foodborne pathogen detection, rapid and accurate detection methods have a significant impact on food safety and public health.

The mean values of the standard plate count for aerobic mesophilic microorganisms, psychrotrophic microorganisms, total coliforms, and *Escherichia coli*, as well as the presence or absence of *Salmonella*, are shown in Table 2.

According to Brazilian microbiological standards for raw beef (maximum 10^6 CFU/g for mesophilic aerobes; 10^2 CFU/g for *E. coli*; absence of *Salmonella* in 25 g), and noting there are no specific limits for psychrotrophs and total coliforms, which are nonetheless important hygienic indicators [18], the analyses indicate the presence of mesophilic aerobic microorganisms in 100% of the samples studied (Table 2). Of these, 66.66% (8/12) present contamination above the maximum limit of 10^6 CFU/g (6 log CFU/g), with average counts ranging from 4.345 to 8.845 log CFU/g. The count of these microorganisms is considered important for determining the microbial load in food, since most pathogenic microorganisms that cause foodborne diseases are mesophilic (20˚C - 45˚C).

Table 2. Average results of microbiological analyses of raw beef sold in supermarkets and street markets in Recôncavo of Bahia, expressed in log UFC/g.

Samples	Mesophiles	Psychrotrophics	Total coliforms	*Escherichia coli*	*Salmonella* spp.
ESTAB1	5173	6032	5235	3835	absence
ESTAB2	4471	4425	4315	1585	absence
ESTAB1	6855	5595	5415	3555	absence
ESTAB2	7689	6434	3274	3033	presence
ESTAB1	6665	6922	5405	3054	absence
ESTAB2	4655	4618	4233	1860	absence
ESTAB1	7395	4035	4265	3235	presence
ESTAB2	8845	5845	5455	3870	presence
ESTAB1	7911	6185	5605	3250	presence
ESTAB2	7595	6275	5325	4933	presence
ESTAB1	6760	4447	4465	3955	absence
ESTAB2	4345	4605	3215	1885	absence

ESTAB1: Open market; ESTAB2: Supermarket.

Similar results were found by Rinn *et al.* [11] and Júnior *et al.* [19], who also described contamination by mesophiles in 100% of meat samples, with 71% of samples showing a contamination level greater than 1.5×10^6 CFU/g in butcher shops in the city of Nampula, Mozambique.

The Heterotrophic Mesophilic Microorganism (HMM) count is used as an indicator of adequate processing conditions, to maintain the cold chain and to predict the shelf life of raw meat [11] [20]. In general, for raw meat, high detection frequencies of HMM between 7 and 8 log CFU/g are associated with lower quality, earlier onset of texture and odor changes, and reduced shelf life [11] [20]. Additionally, high HMM values in raw meat, as found in our study, combined with high detection frequencies of other hygiene-indicating bacteria, such as *E. coli*, indicate unhygienic conditions in the production chain or inadequate temperature during transportation and storage [11].

There was growth of psychrotrophic microorganisms with average values ranging from 4.035 to 6.922 log CFU/g. Although Brazilian legislation does not refer to the total count of psychrotrophic microorganisms, the high count of this group directly influences the shelf life of the product.

Some authors cite psychrotrophic counts below 2 log CFU/g as indicators of low contamination, between 3 and 4 log CFU/g as intermediate, and 5 - 6 log CFU/g as high contamination [11] [21].

Based on this classification, five (41.66%) establishments presented intermediate values and seven (58.33%) presented high contamination. Slightly similar results were described by Velho *et al.* [22], where 37.5% of fresh beef samples sold in supermarkets in Mossoró-RN presented intermediate contamination and 68.5% high contamination, with averages from 3.24 to 6.64 log CFU/g.

As observed in **Table 2**, total coliforms were isolated in 100% of the samples analyzed, with averages ranging from 3.215 to 5.605 log CFU/g. The results found for this group are similar to those presented by Marcanzoni *et al.* [23], who, when analyzing beef sold in Chapecó-SC, also observed a high degree of contamination, with the presence of total coliforms in 100% of the samples.

Regarding the occurrence of *E. coli*, the lowest average values observed, which meet the parameters established by law, correspond to supermarkets in Santo Antônio de Jesus, Muritiba and Santo Amaro, representing 25% (3/12) of the total establishments verified.

An important limitation concerns the restricted number of samples analyzed. Although visits were conducted in all twenty municipalities of the Recôncavo region, with six localities selected as the most representative, it was not possible to increase the number of collections due to safety concerns in the field. A considerable portion of the meat available showed evidence of originating from clandestine slaughter, a practice that not only constitutes a sanitary crime but also poses a significant public health risk by potentially exposing consumers to pathogenic microorganisms. In addition, clandestine slaughter undermines traceability and weakens epidemiological surveillance systems.

This limitation may have influenced the statistical analysis. While the two-way ANOVA showed no significant differences between supermarkets and street markets ($F(1, 40) = 2.58$; $p = 0.1162$), and no interaction between establishment type and microorganism ($p = 0.927$), the restricted number of samples may have reduced the statistical power to detect differences between these factors. On the other hand, highly significant differences were observed among the microorganisms ($F(3, 40) = 19.07$; $p < 0.0001$), and Tukey's post hoc test confirmed that *E. coli* counts were consistently lower than those of mesophiles, psychrotrophs, and total coliforms. Thus, although the reduced sample size limited the ability to explore differences across establishments or municipalities, it was sufficient to demonstrate clear and meaningful distinctions among microbial groups.

This context underscores the urgent need to intensify sanitary inspection measures, ensure stricter enforcement of existing legislation, and develop public policies aimed at eradicating irregular slaughter. At the same time, it reinforces the importance of future studies with larger sample sizes and broader coverage.

In another study, Oliveira *et al.* detected the presence of *E. coli* in 70% and coliforms in 100% of beef samples in João Pessoa-PB, corroborating our findings [24]. According to Alves *et al.*, when present in food, *E. coli* indicates the occurrence of contamination by material of fecal origin, suggesting exposure of the product to unsatisfactory hygienic-sanitary conditions during handling and the possible presence of other enteric pathogens [25].

For *Salmonella*, the legislation establishes the absence of the microorganism in 25 g of raw meat [18]. Therefore, the beef samples sold in the municipalities of Cruz das Almas, São Félix and Cachoeira are considered unfit for consumption. Of these, three come from street markets and two from supermarkets.

Lower values were observed by Oliveira *et al.* in Barra do Garças-MT, where none of the samples analyzed indicated the occurrence of the pathogen [26].

On the other hand, Maia & Oliveira observed *Salmonella* in all beef samples in municipalities in Angola; similar results were found for total coliforms, correlating high contamination with poor display/marketing conditions and inadequate hygiene of handlers [8]. Velho *et al.* also identified the pathogen in 63% of supermarkets and 88% of stalls in Mossoró-RN [22].

The *stx* gene was identified in two of the *E. coli* isolates (**Figure 1**), which came from samples sold at street markets in the cities of Cachoeira and Santo Amaro. Both samples exceeded the maximum legal limits for mesophilic aerobes and *E. coli*; *Salmonella* was also detected in the Cachoeira street market sample. This result was superior to that of Marquezine *et al.*, who did not identify *stx* in 215 beef-cut samples from meat plants in São Paulo [27]. In another study, Stella *et al.*, evaluating swabs from 154 bovine carcasses in Mineiros-GO, also found no *stx* gene [28].

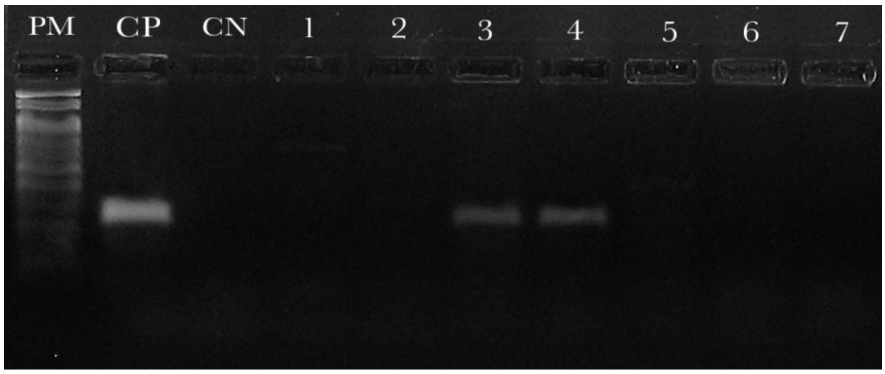

Figure 1. Photograph of the 2% agarose gel of the PCR for the *stx* gene found in *E. coli* samples isolated from beef. Fragment size: 227 bp; MW: Molecular Weight 100 bp; CP: Positive Control; NC: Negative Control; Positive samples: 3 and 4.

Higher numbers were observed by Tarazi *et al.* in northern Jordan: among 194 *E. coli* isolates from human/animal diarrheic feces and retail ground beef, 57 were EHEC (*stx*) [29].

The *stI* gene was not identified among the strains analyzed, and there was no genotypic characterization of enterotoxigenic *E. coli* (ETEC).

Beef with poor sanitary quality sold in Recôncavo of Bahia represents a serious problem with impacts that extend from local trade to state, national, and international levels. Several studies and analyses indicate high levels of contamination by microorganisms such as *Escherichia coli*, *Salmonella*, total coliforms, mesophilic, and psychrotrophic bacteria in samples of beef sold in street markets and supermarkets in the region. This situation highlights significant deficiencies in hygiene practices, maintenance of the cold chain, and proper handling of the product, directly compromising the food safety of consumers.

At the local level, the main consequence is the loss of consumer confidence. When

the population perceives the health risk, they tend to avoid consuming meat from certain points of sale, which directly affects the livelihood of small traders and butchers, especially those who operate in street markets. In addition, the beef produced and sold in Recôncavo is now seen as a product of lower value, economically devaluing what could be an important source of income for the region. Many of these establishments also do not have the necessary structure to meet health requirements, further aggravating the problem.

At the state level, the losses are even greater. The image of the agri-food sector in Bahia is damaged, mainly because this state is one of the main meat producers in the Northeast. When a region systematically presents meat with a high degree of contamination, there is a negative effect on the reputation of the entire state, affecting the acceptance of the product in more demanding consumer centers, such as Salvador and tourist cities. The state health surveillance department is pressured to intensify its actions, which increases operating costs and can generate tensions with local traders. In addition, low-quality meat makes it difficult to sell to other regions of the state, reducing the internal competitiveness of the product.

In the national context, the problem can result in informal trade barriers, especially in distribution networks that value strict quality and traceability criteria. Although meat from the Recôncavo region is not directly included in the export market, its poor health reputation harms meatpacking plants, cooperatives, and local entrepreneurs who wish to expand their markets to other regions of Brazil. There is also a considerable risk to public health, since contaminated meat can contribute to outbreaks of foodborne diseases, generating costs for the health system, lawsuits, and distrust regarding the effectiveness of health inspections.

On the international scene, even though the region's meat is not destined for large-scale export, the growing demand for traceability and food safety in global markets creates an indirect requirement on the entire national production chain. Recurrent cases of contamination in certain regions of the country may compromise Brazil's image as a reliable supplier, even influencing import policies in partner countries. Furthermore, studies conducted in African and Asian countries reveal similar patterns of contamination in contexts of precarious infrastructure, lack of effective inspection, and inadequate handling of meat—showing that the meat health problem is not exclusive to Recôncavo or Brazil, but is part of a global food crisis that requires international cooperation, harmonization of health standards, and strengthening of epidemiological surveillance networks.

Given this scenario, it is urgent to implement public policies and intervention strategies that prioritize the training of handlers, improvements in the infrastructure of points of sale, increased inspection, and incentives for the formalization of the production chain. The problem of low-quality meat in the Recôncavo of Bahia is not only a local challenge, but a reflection of structural weaknesses with interterritorial and international implications, which, if not corrected, will continue to compromise the health of consumers and the competitiveness of the Brazilian production chain on the global stage.

The contamination patterns observed in this study can be explained by a combination of underlying mechanisms along the beef production and retail chain. High counts of aerobic mesophiles reflect both the initial microbial load at slaughter and subsequent deficiencies in hygienic practices during handling, deboning, and distribution. Elevated psychrotrophic counts are strongly associated with failures in maintaining the cold chain, since these microorganisms are capable of growing even under refrigeration, thus reducing shelf life and accelerating spoilage. The consistent detection of total coliforms and *E. coli* indicates fecal contamination, either directly through contact with intestinal content during slaughter or indirectly via cross-contamination from handlers, equipment, or surfaces. Finally, the presence of *Salmonella spp.* in one-third of establishments highlights severe lapses in sanitary control, given that this pathogen should be absent from raw beef according to Brazilian legislation and international standards.

From a public health perspective, these findings are alarming. Consumers exposed to contaminated beef are at increased risk of foodborne diseases, ranging from self-limiting diarrhea to severe conditions such as hemolytic-uremic syndrome, septicemia, and death. Moreover, the occurrence of *E. coli* carrying *stx* genes in some isolates raises concerns about the circulation of Shiga toxin-producing *E. coli* (STEC) in informal production and retail chains, with implications for both human health and epidemiological surveillance. Beyond acute infections, persistent exposure to high microbial loads may also contribute to the dissemination of antimicrobial resistance, exacerbating global challenges in food safety and public health.

At the local level, poor hygienic quality of beef compromises consumer trust and undermines the livelihoods of small-scale vendors, especially those operating in street markets with precarious infrastructure. At the state level, recurrent detection of contaminated meat damages the reputation of Bahia's agri-food sector, creating barriers to distribution to more demanding markets such as Salvador and tourist regions. Nationally, this problem can limit the competitiveness of beef from the Recôncavo, while also increasing public health costs due to foodborne outbreaks. On the international stage, although beef from this region is not primarily destined for export, recurrent sanitary failures can indirectly harm Brazil's image as a reliable food supplier, especially in the context of increasing global demand for traceability and sanitary guarantees.

Altogether, these findings emphasize the urgent need for integrated strategies combining stricter sanitary inspections, effective enforcement of legislation, and the implementation of preventive programs such as Hazard Analysis and Critical Control Points (HACCP). In addition, investments in infrastructure, training of meat handlers, and public education on food safety are essential to mitigate risks. The situation in the Recôncavo region thus illustrates not only a local challenge, but also a structural issue with national and international implications—one that directly links animal production practices, environmental hygiene, and human health, reinforcing the One Health perspective.

4. Conclusions

Given the presence of microorganisms that indicate fecal contamination and are harmful to humans, the high bacterial load in the beef sold in the municipalities of Santo Antônio de Jesus, Cruz das Almas, Muritiba, São Félix, Cachoeira, and Santo Amaro compromises the product's quality and puts consumers' health at risk.

Promoting health education initiatives that seek to educate meat handlers and traders on the value of food safety, personal hygiene, and safe handling techniques is crucial. Additionally, consumers should be aware of the dangers associated with buying and consuming animal-derived products without a provenance.

Then, there needs to be more control over the health inspections that are conducted by the appropriate authorities on the marketing of animal-derived products, as well as the training and qualification of these services, particularly in interior cities and street markets where contamination rates are typically even higher.

As a result, public authorities are crucial in advancing one health through public health-related policies, programs, and laws.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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