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Antimicrobial resistance profile of *Salmonella* spp. isolated from non-edible animal products from slaughterhouses.

Perfil de resistência antimicrobiana de *Salmonella* spp. isolados de produtos de origem animal não comestíveis em abatedouros frigoríficos.

Perfil de resistencia antimicrobiana de *Salmonella* spp. aislados de productos animales no comestibles en mataderos frigoríficos.

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Abstract

Animal origin flours is the non-edible by-product resulting from the processing of waste from the slaughter of animals, not intended for human consumption. In addition to taking advantage of waste, this process aims to reduce environmental damage. However, during some stage of the process of its elaboration may occur contamination by antimicrobials resistant microorganisms such as *Salmonella* spp. When serving as food for these animals, these products can spread pathogens on farms, causing flock infection. Food contaminated with *Salmonella* spp. antimicrobial resistant has a direct negative impact on poultry performance, as well as a risk to consumer health through carcass consumption. Thus, the aim of the present study was to investigate the antimicrobial resistance profile in *Salmonella* spp. isolated from non-edible animal from slaughterhouses located in Bahia and Pernambuco states, Brazil. From biochemical tests for isolation and identification of *Salmonella* spp., 81 isolates were randomly selected to be submitted to antimicrobial susceptibility testing using the plate diffusion method. Most of the isolates was sensitive to the antimicrobials tested. Nalidixic acid showed the highest percentage among those that were resistant, one of the antimicrobials used in the treatment of salmonellosis. This fact can be considered worrying, since the food production chain of animal origin can be an important carrier of resistant strains, especially since it is at the beginning of the production process.
Keywords: Salmonelosis; One health; Food safety; Infection control; Food technology.

Resumo

Farinha de origem animal é o subproduto não comestível resultante do processamento de resíduos do abate de animais de açougue, não destinados ao consumo humano. Além de se aproveitar resíduos esse processo tem o objetivo de

reduzir danos ambientais. No entanto, durante alguma etapa do processo de sua elaboração pode ocorrer contaminação por microrganismos resistentes a antimicrobianos como *Salmonella* spp. que, ao servir de alimento para estes animais, podem disseminar patógenos nas granjas acarretando infecção dos lotes, além de causar impacto negativo direto no desempenho produtivo das aves, bem como risco à saúde do consumidor por meio do consumo dos produtos avícolas. Assim, o objetivo do presente estudo foi investigar o perfil de resistência a antimicrobianos em *Salmonella* spp. isoladas de farinhas de origem animal não comestíveis utilizadas na formulação de rações e também de rações produzidas a partir destes subprodutos de origem animal não comestíveis em abatedouros frigoríficos localizados na Bahia e Pernambuco (Brasil). A partir de testes bioquímicos para isolamento e identificação de *Salmonella* spp. foram selecionados aleatoriamente 81 isolados para serem submetidos a teste de sensibilidade antimicrobiana através do método de difusão em placa. A maioria dos isolados foi sensível aos antimicrobianos testados. Dentre os antimicrobianos que se apresentaram resistentes, o ácido nalidíxico apresentou maior percentual, sendo este um dos antimicrobianos usados no tratamento de salmoneloses. A cadeia de produção de alimentos de origem animal pode ter papel na dispersão de cepas resistentes. Assim, é preocupante o desenvolvimento de resistência a antimicrobianos em microrganismos veiculados por alimentos.
Palavras-chave: Salmonelose; Saúde única; Inocuidade dos alimentos; Controle de infecção; Tecnologia de alimentos.

Resumen
La harina de origen animal es el subproducto no comestible resultante del procesamiento de los desechos del sacrificio de animales de carnicería, no destinados al consumo humano. Además de aprovechar los residuos, este proceso tiene como objetivo reducir el daño ambiental. Sin embargo, durante alguna etapa del proceso de su elaboración, la contaminación por microorganismos resistentes a los antimicrobianos como *Salmonella* spp. que, al servir de alimento a estos animales, pueden propagar patógenos en las granjas provocando la infección de los rebaños, además de causar un impacto negativo directo en el rendimiento productivo de las aves, así como un riesgo para la salud del consumidor a través de el consumo de productos avícolas. Por lo tanto, el objetivo del presente estudio fue investigar el perfil de resistencia antimicrobiana en *Salmonella* spp. aislado de harina animal no comestible utilizada en la formulación de raciones y también de raciones producidas a partir de estos subproductos animales no comestibles en mataderos ubicados en Bahía y Pernambuco (Brasil). A partir de pruebas bioquímicas para aislamiento e identificación de *Salmonella* spp. Se seleccionaron aleatoriamente 81 aislamientos para someterlos a pruebas de susceptibilidad antimicrobiana mediante el método de difusión en placa. La mayoría de los aislados fueron sensibles a los antimicrobianos probados. Entre los antimicrobianos que resultaron resistentes, el ácido nalidíxico presentó el mayor porcentaje, que es uno de los antimicrobianos utilizados en el tratamiento de la salmonelosis. La cadena de producción de alimentos de origen animal puede desempeñar un papel en la propagación de cepas resistentes. Por lo tanto, es preocupante el desarrollo de resistencia antimicrobiana en microorganismos transmitidos por alimentos.
Palabras clave: Salmonelosis; Salud única; Seguridad alimenticia; Control de infección; Tecnología de los Alimentos.

1. Introduction

The slaughter of animals aims to obtain meat and viscera intended for human consumption (Pardi, et al., 2007). With the exception of the muscle parts, which are considered of greater economic value, the other parts derived from the slaughter process are considered by-products (Alao, et. al., 2017) and are classified into edible and non-edible by-products (Okanović, et al., 2009; Jayathilakan, et al., 2012; Mathi, et al., 2016; Fayemi, et al., 2018; Malav, et al., 2018). One of the ways to take advantage of non-edible waste generated in the animals slaughter is the production of animal origin flours. (Jayathilakan, et al., 2012; Thyagarajan, et al., 2013). The process involves stages of grinding, mixing, pressing, decanting and separation. The obtained solid is then is ground into flour, followed by thermal processing by cooking, evaporation or drying, as well as chemical processing, such as solvent extraction (Malav, et al., 2018). These residues have a considerable microbiological composition that, if not stabilized, can lead to decomposition and environmental impacts (Pacheco, 2006; Jayathilakan, et al., 2012).

Despite the use of high temperature in treatment of animal origin flour, many microbiological contaminants has the capacity of thermoresistance. Thus, the final product can yet present viable microorganisms, among them *Salmonella* spp. (Liu, et al., 2018) which is an important cause of foodborne diseases in the world (Pal, et al., 2015; Hunter, et al., 2017). Some serotypes are considered host-specific, although the degree of adaptability varies by serotype and affects the pathogenicity of humans and animals (Evangelopoulou, et al., 2013; Singh, 2013; Andino & Hanning, 2015). However, most serovars are not

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host-specific and can infect several animal species, including humans and all belong to the *enteric* subspecies (Uzzau, et al, 2000; Andino & Hanning, 2015). These are most often involved in foodborne diseases, with those of animal origin being the main ones (Da Silva, et al., 2017). In addition, this bacterium stands out for its multi-resistance to antimicrobials (Chen, et al., 2013; Silva, et al., 2014; Tegegne, 2019).

The existence and spread of antimicrobial-resistant microorganisms are the result of the selection pressure imposed by man, the unnecessary prescription of drugs, the incorrect use in treatments without diagnosis, self-medication, disposal of antimicrobial residues in the environment, or by the use of these in the livestock as growth promoters (Guimarães, et al., 2010; Marshall & Levy, 2011; Khojasteh, et al., 2018). According to the Brazilian National Health Surveillance Agency, about 50% of medical prescriptions for these drugs are made inappropriately and, therefore, one of the goals of the World Health Organization (WHO) for the 21st century is the conscious use of antimicrobial agents, thus preventing the emergence and selection of resistant strains (Brasil, 2007). The increase in infections caused by antimicrobial-resistant bacteria is accompanied by a decrease in the effectiveness of treatment with these drugs (Frye & Jackson, 2013; Khojasteh, et al., 2018).

Mota, et al. (2005) also add that only 50% of the antibiotics produced are used in human therapy, the other half is used in prophylaxis, treatment, or as animal growth promoters, and in the extermination of pests in agriculture. In animal husbandry, the emergence of resistant microorganisms can also occur through the use of antimicrobials in the feed (Yates, et al., 2004). During the last 70 years, the abusive use of antimicrobials in human medicine and in animal husbandry has led to a steady increase in microorganisms resistant to these drugs (World Health Organization, 2012). Although there is a consensus among the scientific community about the consequences for human health due to the use of antimicrobials in animal production, the same is not true regarding the proportion of antimicrobial resistance in bacteria isolated from humans caused by the use of antimicrobials in this production system (World Health Organization, 2017). It is estimated that about 63,151 tons of antimicrobials are used in livestock worldwide alone, with a growth forecast of 67% between 2010 and 2030, making this sector the main user of antimicrobials, including in poultry (Van Boeckel, et al., 2015).

The poultry industry has an important contribution to the wide dissemination of *Salmonella* spp. clones drugs in several countries (Bada-Alambedji, 2006; Antunes, et al., 2016; Sanchez-Salazar, et al., 2020). According to Gebreyes & Thakur (2005), *Salmonella* spp. was once considered the largest reservoir of antimicrobial resistance in humans and animals. In studies carried out by these researchers, 75% of the swine isolates showed resistance to seven antimicrobials while one human isolate showed resistance to 10 of the 12 antimicrobials tested. The spread of these multidrug-resistant strains through the food chain represents an important public health problem, considering the potential for failures during the treatment of infected people if their use becomes necessary (Folster, et al., 2010; Liljebjelke, et al., 2017; World Health Organization, 2017). Thus, the objective of this study was to investigate the antimicrobial resistance profile of *Salmonella* spp. isolated from non-edible animal products intended for poultry feeding and also of feed produced from these by-products in in slaughterhouses samples received from Bahia and Pernambuco states, Brazil.

2. Methodology

From *Salmonella* spp. isolated and identificated from flour intended for the preparation of meal samples and rations samples intended for feeding broilers, the technique was carried out to determine the sensitivity profile to antimicrobials through the agar diffusion test, using the methodology described by Bauer, et al. (1966). The isolates of *Salmonella* were added in 3 mL of BHI (Brain Heart Infusion) broth and incubated at 35°C for 24 hours. A few drops of bacterial growth were then transferred to tubes containing 3 mL of sterile 0.85% saline solution until reaching a turbidity corresponding to 0.5 on the Mac Farland scale (approximately 1.5×10^8 Colony-forming unit/mL). Then, with the aid of a sterile swab, the dilution was seeded on plates containing Müller-Hinton (Kasvi®) agar using the surface scattering technique, in order to obtain homogeneous

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bacterial growth throughout its extension. After dissemination, with the aid of previously sterilized tweezer, the discs containing the antimicrobials (Laborclin®; CECON®) to be tested were applied to the plates. For each sample, two plates were used, each containing five or six disks, organized so that the distance between them does not interfere with the inhibition zone of another tested antimicrobial agent. The plates were incubated upside down at 35°C ± 2°C for 18 hours. At the end of this period, they were performed by determining the diameters of the inhibition halos of bacterial growth, measured with the aid of a halometer. The interpretation of the results obtained regarding the sensitivity profile against the antimicrobials tested was based on the table of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2020). The profiles presented by the *Salmonella* spp. studied were classified as sensitive (S), intermediate (I) or resistant (R). The antimicrobials tested in the present work were from the Beta-lactam groups: amoxicillin/clavulamic acid (20/10μg), ceftazidime (30μg), cefotaxime (30μg), ceftriaxone (30μg), (10μg); tetracycline (30μg); sulfonamides: sulfal/trimethoprim (25μg); aminoglycoside: gentamicin (10μg); fluoroquinolones: ciprofloxacin (5μg), nalidixic acid (30μg) and norfloxacin (10μg). The choice of antimicrobials was based on their importance both in veterinary medicine, due to their frequent use, and in human medicine, as they are treatment options (Clinical and Laboratory Standards Institute, 2020).

The data was analyzed using the SAS/STAT® software, version 9.1 (2004). Chi-square test was used to evaluate antibiotic resistance of *Salmonella* spp., isolated from non-edible animal products from slaughterhouses in Bahia and Pernambuco states in Brazil.

3. Results and Discussion

Most of the isolates was sensitive to the antimicrobials used in the research. Table 1 shows the interpretation of the resistance profile presented by the 81 isolates of *Salmonella* spp. of non-edible animal products. Of the 10 antimicrobials tested, a resistance profile, was observed for the following antimicrobials: amoxicillin (3.3%), cefotaxime (1.2%), gentamicin (2.5%) ceftazidime (1.2%), ceftrizxone (1.2%) and ciprofloxacin (2.5%).

Nalidixic acid showed the highest level of resistance among the isolates (33.3%), corroborating other similar studies where the profile of antimicrobial resistance in *Salmonella* spp. nalidixic acid showed higher resistance patterns as Duarte, et al., (2009) and Pandini, et al., (2015) with 21% and 28.2%, respectively. All isolates in this study were 100% susceptible to the antimicrobials norfloxacin, tetracycline and sufamethoxazole+trimethoprim. Similar results were found by De Souza, et al. (2010) and by Pandini, et al. (2015) when evaluating the resistance profile of *Salmonella* spp. isolated from aviaries in other Brazilian state. In both study all isolates showed susceptibility to the antimicrobial norfloxacin.

Table 1: Antimicrobial resistance/susceptibility profile of *Salmonella* spp. Isolated from samples of inedible animal products.

PROFILE	ANTIMICROBIALS									
	AMC	NAL	CTX	GEN	NOR	TET	CAZ	CRO	SUT	CIP
	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)	% (n)
Sensitive	96.3 (78)	66.7 (54)	98.8 (80)	96.4 (78)	100.0 (81)	100.0 (81)	96.4 (78)	98.8 (80)	100.0 (81)	84.0 (68)
Intermediate	0.0 (0)	0.0 (0)	0.0 (0)	1.2 (1)	0.0 (0)	0.0 (0)	2.5 (2)	0.0 (0)	0.0 (0)	13.5 (11)
Resistant	3.7 (3)	33.3 (27)	1.2 (1)	2.5 (2)	0.0 (0)	0.0 (0)	1.2 (1)	1.2 (1)	0.0 (0)	2.5 (2)
Total isolates	100 (81)	100 (81)	100 (81)	100 (81)	100 (81)	100 (81)	100 (81)	100 (81)	100 (81)	100 (81)

AMC =Amoxicillin/clavulamic acid (20/10 µg); NAL = Nalidixic acid (30 µg); CTX = Cefotaxime (30 µg); GEN = Gentamicin (10 µg); NOR = Norfloxacin (10 µg); TET =Tetracycline (30 µg); CAZ = Ceftazidime (30 µg); CRO = Ceftriaxone (30 µg); SUT = Sufamethoxazole/trimethoprim (25 µg); CIP = Ciprofloxacin (5 µg). Source: Authors.

No antibiotic resistance was observed for norfloxacin, tetraciclin and sufamethoxazole + trimethoprim. A slight number of samples showed small resistance to cefotaxime, ceftriaxone, amoxicillin + clavulamic acid, gentamicin, ceftazidime and ciprofloxacin. However, the highest resistance observed was for nalidixic acid. Nalidixic acid was also the only significantly drug resistance observed in the group of antibiotics evaluated ($p<0.05$).

The minimum inhibitory concentration (MIC50) is an important indicator for *in vitro* antimicrobial activity. Though these values are not necessarily predictive of antimicrobial agent activity *in vivo* (De Souza, et al., 2010). The use of antimicrobials in veterinary medicine in a prophylactic way or as growth promoters can facilitate the selection of resistant bacteria or the reduction of susceptibility to these antimicrobials. It is believed that the use of these antimicrobials in production animals is likely the cause of the emergence of these resistant strains (Robles-Jimenez, et al., 2021). According to Hooper (2001), two decades after the use of fluoroquinolones, the bacteria began to show resistance. The isolates featured in this research showed 100% sensitivity to norfloxacin, one of the three antimicrobials of the fluoroquinolone group tested. While nalidixic acid, also from the same group, showed the highest percentage of resistant isolates.

Flouorquinolone resistance is linked to mutations developed in the fluoroquinolone resistance-determining regions (QRDR) of the gyrA and ParE genes that encode DNA-gyrase and DNA-topoisomerase IV enzymes, respectively, which represent targets of action of fluoroquinolones (Hooper & Jacoby, 2015). A single point mutation is required to determine resistance to certain fluoroquinolones such as nalidixic acid which are first generation, while others such as ciprofloxacin need additional mutations. In addition to the fluoroquinolones norfloxacin, tetracycline and sulfamethoxazole+trimethoprim showed 100% susceptibility. Fluoroquinolones are among the main antimicrobials used in the treatment of infections by *Salmonella* spp. in adults, while third-generation cephalosporins are used to treat infections in children or the elderly (European Food Safety Authority, 2014).

Although there is an increase in the use of β-lactams worldwide in the treatment of Gram negative bacteria (Pitout & Laupland, 2008), in this study it was possible to verify that the tested isolates showed a low resistance profile to the antimicrobials of this class used (amoxicillin + clavulamic acid, ceftiazidime, ceftriazone and ceftiofur). Of the 10 antimicrobials tested, the second one with the highest resistant profile was amoxicillin with calvulamic acid (3.7%) followed by cephalosporins such as cefotaxime, ceftazidime and ceftriaxone, which showed 1.2% resistance. Most antimicrobials proved to be efficient against *Salmonella* spp. analyzed. With the exception of nalidixic acid, all strains tested presented a high percentage of sensitivity, above 80%: amoxicillin with clavulamic acid (96.3%), cefotaxime (98.8%), gentamicin (96.4%), ceftadizime (96.4%), ceftriaxone (98.8%) and ciprofloxacin (84%).

The resistance pattern of *Salmonella* spp. presented was higher against nalidixic acid, an antimicrobial used in the treatment of human infections caused by this pathogen (Rodrigues-Silva, et al., 2014). This raises greater concern for the possibility of resistance generation during the treatment of patients with infections of this type (Mølbak, 2004). The acquisition of antimicrobial resistance may vary from one serotype to another. In general, *Salmonella* Typhimurium has shown higher resistance profiles to the most commonly used antimicrobials (Ahmed, et al., 2016; Borges et al., 2019). Liljebjelke, et al. (2017) report that antimicrobial-resistant *Salmonella* Typhimurium isolates would likely be transmitted vertically on farms where they were confined before slaughter, as the strains isolated in carcasses and chicks housed after the same breeder are the same.

In the United States *Salmonella* spp. isolated between 2016 and 2017 showed increased resistance to third-generation cephalosporins, fluoroquinolones or azithromycins. This is a cause for concern, as these are among the antimicrobials considered to be on the front line in the treatment of complications in infections caused by this pathogen. In the same period, no resistance to carbapenems was found in isolates of this pathogen from humans, animals or food. This is an important fact, as this class of antimicrobials is used to control multidrug-resistant strains in this country (United States Food And Drug Administration, 2019).

The increase in resistance to cephalosporins in *Salmonella* Heidelberg isolates was determined by the dissemination of the plasmid encoded by the blaCMY gene, and the identification of identical sequences encoded by the IncI1 plasmid in strains isolated from humans, poultry carcasses from slaughterhouses and chicken breasts sold in retail markets. This supports the evidence that poultry products are an important source of infection for humans (Folster, et al., 2012). A study on resistance to cephalosporins in *Salmonella* Typhimurium strains showed that while most isolates had the plasmid encoded by the blaCMY gene, almost all isolates from chickens had the plasmid IncI1-blaCMY, while those from cattle had the plasmid IncA/CblaCMY (Folster, et al., 2014).

Another important factor to be considered is that flours made with non-edible animal products used in the preparation of poultry feed can represent a source of infection for humans (Morente, et al., 2013). Whereas *Salmonella* spp. antimicrobial resistance can be transmitted to humans through food consumption (Frye & Jackson, 2013, Alam, et al., 2019). There are several studies confirming the link between the incidence of resistant bacteria in both humans and animals and production with a risk of developing infections, hospitalizations and deaths, including those caused by *Salmonella* spp. (Helms, et al., 2003; Molbak, 2004; Helms, et al., 2006). Even with several routes of exposure to infections such as water, food, humans and even animals, the consumption of food of animal origin is still the most important vehicle of transmission (Morente, et al., 2013).

This low resistance index may indicate a reduction in the use of antimicrobials indiscriminately in poultry farming as growth promoters, in a prophylactic or even therapeutic way (Salim, et al., 2018). Although many studies warn of the emergence of multi-resistance strains due to the indiscriminate use of this type of drug in humans and animals (Borges, et al., 2019; Asfaw, Ali et al., 2020; World Health Organization, 2020). This can lead to unique health impacts, directly by interfering with the patient's treatment or indirectly by the possibility of dissemination of resistance factors among pathogens (Frye & Jackson, 2013).

The Brazilian Ministry of Agriculture, Livestock and Supply implemented the Program for Surveillance and Monitoring of Resistance to Antimicrobials in the Scope of Agriculture, which aims at risks, trends and patterns in the occurrence and dissemination of resistance to antimicrobials through food of animal origin produced in Brazil, as well as provide essential data for risk analysis relevant to animal and human health (Brasil, 2021). The analysis of this type of behavior would be important because it allows the adoption of measures to control and restrict the use of antimicrobials (Borges, et al., 2019).

4. Conclusion

Worldwide, antimicrobial resistance seems to increase in several commensal or pathogenic microorganisms, including those transmitted by food. Although the isolates found in this research showed sensitivity to most of the antimicrobials used, the considerable resistance to nalidixic acid, an antimicrobial used in the treatment of human infections by this pathogen is worrying, since the food production chain of animal origin can be an important carrier of resistant strains, mainly because it is at the beginning of the production process. Future work needs to be developed to evaluate the association of infections in humans and animals attributed to multidrug-resistant strains as well as a possible transmission through food consumption.

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