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Occurrence of *Escherichia coli* in salmon *sashimis* commercialized in restaurants from Londrina - PR

Ocorrência de *Escherichia coli* em *sashimis* de salmão comercializados em restaurantes de Londrina - PR

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Abstract

The population interest for fish consumption has increased, mainly due to several beneficial nutritional properties presented by this food. In this context, oriental culinary also brings different eating habits as consume raw food, such as *sashimi*. A relevant food contaminant of fecal origin is *Escherichia coli*, able to become potentially harmful, when it acquires virulence factors, as Shiga toxin-producing *E. coli* (STEC). This study aimed to evaluate 30 samples of salmon *sashimi* regarding the presence of *E. coli*, as well as perform the genotypic characterization of virulence factors associated with STEC. Three samples were collected from 10 different restaurants, specialized in Japanese culinary in the city of Londrina - PR. The *E. coli* identification was performed using the Colilert® chromogenic substrate technique and biochemical tests, and for the investigation of virulence genes, *stx1* and *stx2*, the polymerase chain reaction (PCR) was used. Among the 30 samples analyzed, 15 (50%) presented contamination by *E. coli*. However, in no sample were detected virulence factors associated with STEC. Although human diseases associated with STEC are poorly described in Brazil, it is possible to verify that fish, mainly those consumed raw, are potential transmitters of *E. coli* to humans. This can compromise the food safety of these products and, thus, characterize them as unsuitable for consumption. Therefore, it is necessary the adoption of preventive measures of contamination by *E. coli* in products intended to human consumption, beyond more research that can verify the potential of STEC as a fish contaminant.

Keywords: *E. coli*. Salmon. Japanese culinary. Microbial contamination. Food safety.

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Resumo

O interesse da população pelo consumo de peixe tem aumentado, principalmente devido às diversas propriedades nutricionais benéficas apresentadas por este alimento. Neste contexto, a culinária oriental também traz diferentes hábitos alimentares, como consumir alimentos crus, tais como o *sashimi*. Um relevante contaminante alimentar de origem fecal é *Escherichia coli*, capaz de tornar-se potencialmente danosa ao adquirir fatores de virulência, como *E. coli* produtora de toxina Shiga (STEC). Este estudo objetivou avaliar 30 amostras de *sashimi* de salmão quanto à presença de *E. coli,* bem como realizar a caracterização genotípica de fatores de virulência associados com STEC. Três amostras foram coletadas de 10 diferentes restaurantes especializados em culinária japonesa da cidade de Londrina - PR. A identificação de *E. coli* foi realizada utilizando a técnica de substrato cromogênico Colilert® e testes bioquímicos, e para a investigação dos genes de virulência, *stx1* e *stx2*, a reação em cadeia da polimerase (PCR) foi utilizada. Dentre as 30 amostras analisadas, 15 (50%) apresentaram contaminação por *E. coli*. Contudo, em nenhuma das amostras foram detectados fatores de virulência associados com STEC. Embora as doenças humanas associadas com STEC sejam pouco descritas no Brasil, é possível verificar que os peixes, principalmente aqueles consumidos crus, são potenciais transmissores de *E. coli* aos humanos. Isto pode comprometer a segurança alimentar destes produtos e, assim, caracterizá-los como impróprios para o consumo. Portanto, é necessária a adoção de medidas preventivas de contaminação por *E. coli* nos produtos destinados ao consumo humano, além de mais pesquisas que possam verificar o potencial de STEC como um contaminante de peixes.

Palavras-chave: *E. coli*. Salmão. Culinária japonesa. Contaminação microbiana. Segurança alimentar.

Introduction

The population interest for fish consumption has increased due to the association of its ingestion with improvement in health. This food presents several beneficial nutritional properties, as the high biological value of proteins, high levels of micronutrients, low levels of saturated fat and high level of polyunsaturated fatty acids, as omega-3.[1-3]

Among the fish, salmon *in natura* in the form of *sushi* or *sashimi* is widely consumed in Brazil due the popularization of Japanese culinary. The salmon is rich in liposoluble vitamins (A and D) and minerals such as calcium, copper, iron, phosphorus, magnesium, manganese, selenium and zinc.[1,3]

However, as *sashimi* is consumed raw, not receiving heat treatment able to eliminate or minimize pathogenic microorganisms, it is considered a food with potential risk to consumer health.[4] Thus, the consumption of *sashimi* becomes a concern for public health, not only because it is considered a highly perishable food, but also due to the hygienic-sanitary conditions in which it is prepared and preserved.[5]

The number of foodborne diseases is increasing significantly and among their etiological agents, bacteria are the most important group.[6] The Shiga toxin-producing *Escherichia coli* (STEC) strain has its pathogenicity related to the ability to adhering the intestinal mucosa and produce toxins. The food contamination by STEC can cause complications, such as, the hemolytic uremic syndrome and hemorrhagic colitis.[7] The increased raw fish consumption and the hygienic-sanitary conditions involved throughout the production process are directly associated to contamination and the possibility of infection by *E. coli* strains, presenting virulence factors of STEC pathotype.

Although diarrheal diseases in humans associated with STEC have not been frequently reported in Brazil, national studies in cattle evidence the prevalence of this strain in the country, as well as correlation between serotypes found in these animals and in human patients.[8] The occurrence of STEC in Brazil was investigated, being that its positivity varied from 1.4 to 71% in cattle, from 0 to 18.1% in food sources of animal origin and water and from 0.6 to 6.3% in human clinical

samples. Significant differences in the isolation rates and serotypes were observed.[9] In addition, Brazil borders Argentina, where 80% of the meat consumed is not properly controlled or inspected, justifying the high incidence of STEC infections.[10] In Brazil, the information available on the subject is still scarce, although are recognized sporadic cases of diarrhea by STEC, which occur more frequently in children.[11] Studies performed in the states of São Paulo and Paraná using feces from children with diarrhea report a frequency of STEC of approximately 1%, however there are no reports of outbreaks associated involving STEC in the country.[12-13]

There are some documents that have compiled global STEC data based on sites of health institutions and overview by continent. However, the prevalence and distribution of STEC in Brazil remains unclear. This study aimed to evaluate 30 samples of salmon *sashimi* sold in specialized restaurants of Japanese culinary in the city of Londrina, north of Paraná, south of Brazil, regarding the presence of *E. coli*, as well as perform the genotypic characterization of virulence factors associated with STEC.

Material and Methods

Sampling and prepare of the samples

Thirty samples of salmon fish *sashimi* were collected, being three samples from 10 different restaurants, identified as follows: R1-R10, all ten establishments specialized in Japanese culinary in the city of Londrina - PR, during the period of December 2016. After the purchase through of self-service or table service, the samples collected of balcony or table were packed in sterile bags in isothermal boxes with ice and transported to the laboratory for analysis within 6 h.

Aseptically, 25 g of each sample were weighed in a semi-analytical balance and later diluted in 225 mL of 1% peptone water and homogenized for 20 min.

Isolation and identification of *E. coli*

E. coli identification was performed using the Colilert® (Sovereign - USA) chromogenic substrate technique, which can also be applied to different food matrices as suggested by Oliveira.[14] According to this technique, as described by Schuroff *et al*.,[15] 100 mL of each sample was poured into a sterile bottle and transferred to the *Quanti-Tray* (WP2000) and the Colilert® reagent added. After homogenization, it was used the sealer *Quanti-Tray Sealer* (IDEXX/Sovereign - USA). Then, *Quanti-Trays* were incubated at 37 ºC ± 2 ºC for 24 h and examined under UV light at 365 nm. The yellow wells that become fluorescent blue indicated the presence of *E. coli*.

Aliquots of the fluorescent wells were collected with sterile syringe and transferred to the Broth *E. coli* at 35 ºC for 24 h. After this period, the samples were streaked onto MacConkey Agar and incubated under the same conditions. Presumptive *E. coli* colonies were selected and submitted to biochemical screening using the EPM, MILi and Simmons Citrate (Difco™). Biochemically identified *E. coli* isolates were stored in stock agar at room temperature and at -20 ºC in Brain Heart Infusion (BHI) broth with 20% (v/v) glycerol.

For the search of serotype O157:H7, the samples were streaked onto sorbitol MacConkey Agar and those with typical characteristics were submitted to biochemical identification.

Search for virulence genes

All isolates were screened for the presence of virulence genes, *stx1* and *stx2*, using PCR, as described by Paton and Paton (Table 1).[16] Initially, to extraction of bacterial DNA, *E. coli* isolates were grown in Luria-Bertani Agar at 37 ºC for 24 h. The cultures were suspended in 300 µL of sterile ultrapure water. The bacteria were lysed by boiling for 10 min in a water bath (100 °C).[17] Posteriorly, they were cooled in ice for 5 min and centrifuged at 1000 rpm for the same period.

The PCR was performed in a Biocycler® thermocycler, in 25 µL reaction volume, containing 2 µL of DNA sample, 200 µM of dNTP's, 1.5 mM of $MgCl_2$, 20 pmol of each primer and 1.5 U of Taq DNA polymerase (Invitrogen™). PCR amplification products were subjected to electrophoresis on a 1.5% agarose gel, stained with SYBR® Safe and visualized under UV light. EDL933 (*E. coli* O157:H7) and HB101 (*E. coli* K-12) were used as positive and negative controls, respectively.[15]

Table 1 - Primers used for PCR in the present study.

Gene	Primer Sequence (5´➔3´)	Amplicon size (pb)	Annealing temp. (ºC)	Reference
stx1	(F) ATAAATCGCCATTCGTTGACTAC (R) AGAACGCCCACTGAGATCATC	180	60 ºC	Paton; Paton, 1998[16]
stx2	(F) GGCACTGTCTGAAACTGCTCC (R) TCGCCAGTTATCTGACATTCTG	255	60 ºC	Paton; Paton, 1998[16]

Source: Authors

Results and Discussion

In this study, the biochemical tests confirmed 100% of the bacteria presumptively identified as *E. coli* by Colilert®, however strains of *E. coli* O157:H7 were not found. Among the 30 samples analyzed, 15 (50%) presented contamination by *E. coli* (Table 2), highlighting the potential of fishes in the transmission of this microorganism to humans.

Table 2 - Search results for the identification of *E. coli* in *sashimi* samples.

Restaurants	*E. coli**
R1	Absent
R2	Absent
R3	Present
R4	Absent
R5	Present
R6	Absent
R7	Present
R8	Present
R9	Absent
R10	Present

*Result of three concordant samples analyzed from each restaurant.
Source: Authors

Similarly, Freire *et al*.[18] collected salmon fish *sashimis* on specialized and non-specialized establishments in oriental culinary, commercialized in the city of Mossoró, state of Rio Grande do Norte, Brazil, where from 12 analyzed samples, six were contaminated by *E. coli*.

Moura Filho *et al*.[19] evaluated the microbiological quality of *sashimis* and vegetables that accompanied them in the restaurants of Recife, Pernambuco, Brazil. They detected *E. coli* in 10 of the 30 samples analyzed, being 2 *sashimis* and 8 vegetables. According to Cardozo *et al*.,[20] *E. coli* does not belong to fish gut microbiota. Then, when it is found in these animals, it can be relatively linked to the contaminated water where these fish live, becoming a source of transmission of this pathogen.

Barbosa *et al*.[21] identified 115 *E. coli* isolates, 19 were obtained from water, and 96 were obtained from fish. Regarding fish isolates, 26 were recovered from the skin, 65 from the gastrointestinal tract and five from the muscle. Based on the analysis, water, skin and the gastrointestinal tract presented a higher correspondence and a higher number of isolates; thus, they are possible means for the transmission of *E. coli* serotypes that cause diarrhea. In addition, the water used for ice production is also considered critical factor. Ferreira *et al*.[22] analyzed eight ice samples used in fish conservation and found that six (75%) were contaminated by total and thermotolerant coliforms, and two (25%) by *E. coli*, indicating poor hygienic-sanitary quality of this product.

The quality of fresh fish may also be influenced by manipulators non-hygienic habits. The *sashimi* is a food that is in constant manipulation and does not receive previous heat treatment able to eliminate or at least minimize the risk of possible contaminant microorganisms.[23]

Prado *et al*.[24] analyzed a *sushibar* observing inadequate hygienic procedures, such as the non-control of the binomial time and temperature, inadequate cleaning of hands, fruits and vegetables, utensils and equipment, besides the lack of *sushiman* training, being that the training and supervision of these professionals are important preventive measures.

Another relevant factor is the health of food handlers, that is, everyone who can come into contact with the product, at any food chain stage, play an important role in preparations quality, being the health and hygiene of these professionals directly related to food safety.[25]

E. coli is an important food contaminant and when acquires specific virulence factors, it becomes potentially harmful, as is the case of STEC.[26] Carvalho *et al*.[27] highlighted among the STEC transmission forms the ingestion of raw or undercooked meat, being the *sashimi* important contamination source. Despite its importance as a food contaminant, in the present study, the STEC virulence factors investigated were not detected in any of the *E. coli* isolates (Table 3).

Table 3 - Result of genotypic characterization of virulence factors associated with STEC.

Restaurants	*stx1**	*stx2**
R3	Negative	Negative
R5	Negative	Negative
R7	Negative	Negative
R8	Negative	Negative
R10	Negative	Negative

*Result of three concordant samples analyzed from each restaurant.
Source: Authors

However, Cardozo *et al*.[20] investigated the presence of pathotypes STEC and EPEC (enteropathogenic *E. coli)* in farmed fish and free-living fish. From 373 analyzed samples from the fish farm, one (0.2%) tested positive for a STEC related gene and of the 99 free-living fish analyzed

samples, six (6%) were positive for at least one of the STEC or EPEC related genes, demonstrating how these fishes contribute to humans infections. Kumar *et al*.[28] analyzed fresh seafood and meat marketed in the city of Mangalore, Karnataka, India and genes of STEC were found in two of the 60 fish samples and two of the 48 clam samples.

Finally, although the investigation here reported does not represent a sufficient number of samples to make the results conclusive, studies reporting salmon contamination by STEC are scarce. Recently, in the study of Ramires *et al*.[29] was evaluated the microbiological quality of salmon *sushi* samples, in the city of Pelotas - RS, Brazil. Interestingly, of the 28 samples examined, just one was found to be positive for *E. coli*; however, this isolate was characterized as *E. coli* O157:H7. This isolate presented virulence genes *stx1*, *stx2*, *eae* and *hlyA* and had the ability to form biofilms on stainless steel, suggesting the possibility of persistent contamination throughout the production process, since this is the material most used in food-processing and storage environments.

Conclusion

Sashimis prepared in establishments specialized in Japanese culinary can present risks to consumer health, since they can be contaminated by *E. coli*. This microorganism may indicate inappropriate contamination of raw material or contamination during food preparation as well as inadequate sanitation or cleaning practices. Pursuant to these results and the increase in the consumption of raw salmon, there is a need to improve the good hygiene practices adopted in establishments selling *sashimi* to minimize the risk of contamination by deteriorating or pathogenic bacteria that may cause harm to the health of consumers.

Regarding the research of STEC virulence factors, a wider study of the topic is essential, since most studies reported STEC contamination in foods of bovine origin, thus hampering a conclusive investigation of the contamination of fish in relation to STEC.

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