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Microbiological quality of "Doner kebab" sold in retail in Sao Paulo- Brazil

Qualidade Microbiológica do Churrasco grego (Doner Kebab) vendido no varejo em São Paulo – Brazil

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ABSTRACT
Most part of Brazilians does not usually make meals in their homes due to the bustling routine. The kebab (consisting of bread, meat and vinaigrette), marketed mainly in the downtown of São Paulo, is a good choice because of speed of preparation and low cost but, street foods stand out for the frequency of foodborne illness outbreaks. According to Brazilian legislation, are allowed to 10^2/g of thermotolerant coliforms (TtC), coagulase-positive staphylococci 10^3/g of (CPS) and sulfite-reducing Clostridium (SRC) in the absence of Salmonella spp., in 25 grams of the product. The aim of this study was to evaluate the microbiological quality of kebab marketed in downtown of São Paulo, noting whether they are within those standards. A hundred samples were analyzed, from 10 snack bars located in this region, processed and subjected to analysis according to APHA (2001). 1% had Salmonella spp., 12% contained SRC, but only 4% out of the allowed amount, 70% had TtC above these standards and 5% had CPE, but within the allowed parameters. Altogether, 71% of the samples were outside the parameters. These results indicate health risk to consumers, showing also the need to develop SOPs to improve the hygienic-health aspects.

Keywords: Salmonella, Staphylococci, Clostridium, thermotolerant coliforms.

ABSTRACT
A maioria dos brasileiros não costuma fazer refeições em suas casas devido à rotina movimentada. O kebab (composto por pão, carne e vinagrete), comercializado principalmente no centro de São Paulo, é uma boa opção devido à rapidez da preparação e ao baixo custo, mas os alimentos de rua se destacam pela frequência de surtos de doenças transmitidas por alimentos. De acordo com a legislação brasileira, são permitidos 10^2 / g de coliformes termotolerantes (TtC), estafilococos coagulase-positivos 10^3 / g de (CPS) e Clostridium redutor de sulfito (SRC) na ausência de Salmonella spp., Em 25 gramas produtos. O objetivo deste estudo foi avaliar a qualidade microbiológica dos kebab comercializados no centro de São Paulo, observando se eles estão dentro desses padrões. Foram analisadas cem amostras, de 10 lanchonetes localizadas nessa região, processadas e submetidas à análise de acordo com a APHA (2001). 1% tinha Salmonella spp., 12% continha SRC, mas apenas 4% da quantidade permitida, 70% tinham TtC acima desses padrões e 5% tinham CPE, mas dentro dos parâmetros permitidos. No total, 71% das amostras estavam fora dos parâmetros. Esses resultados indicam riscos à saúde dos consumidores, mostrando também a necessidade de desenvolver POPs para melhorar os aspectos de higiene e saúde.

Keywords: Salmonella, estafilococos, Clostridium, coliformes termotolerantes.

1 INTRODUCTION

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Due to the bustling routing, long distance between home and job and short period to have lunch, most part of Brazilian does not usually make meals in their homes. According to ABERC[1] (Brazilian Association of Collective Meals Companies), around 19 million of people made meals outside the home.

Establishments that make and sell snacks, as kebab, stand out in food-borne outbreaks, with high prevalence in developing countries[2].

For reasons of hygiene and health, it is important to have control of all process steps: raw material, conservation, manipulation, transport, storage, prepare and food distribution[3].

The döner kebab (or kebap), known in Brazil as "churrasco grego", was originated in Turkey. It became an ordinary meal in Turkey, Greece, Mediterranean coast (such as South of France) and places with strong Turkish immigration, for example, the main cities of Germany and Austria, besides being consumed a lot in downtown of São Paulo. The kebab is made of slices of grilled meat stacked in a vertical stick that spins.

Salmonella spp., *Escherichia coli*, *Staphylococcus aureus* and *Clostridium perfringes* can be found in a substantial number in kebab and they are an important risk factor to public health[4].

Staphylococcus aureus are usually found in human microbiota (nose, armpit, groin area) and can cause opportunist infection. The main virulence factor is the ability to produce endotoxins, which can cause intoxication through contaminated food consume, due to the pre-formed thermostable enterotoxins. One of the most contaminated foods is the processed meat and this contamination usually comes from the food handlers[10]. The most common symptoms, which generally appear in 4 hours after the food ingestion, are abdominal cramps, nausea, diarrhea, headache, sweating, restlessness and, sometimes, drop of body temperature[11].

Salmonella spp. are Gram-negative, flagellated, facultative anaerobic bacilli, lactose-negative. They can cause gastroenteritis, an intestinal mucosa acute infection, due to their presence in food of animal origin, such as chicken, eggs, rare meat and the main symptoms are headache, fever, chill and abdominal pain. The incubation period of *Salmonella*, not Typhi-type, is on a average of 48 hours[10].

Clostridium perfringens are Gram-positive, rod-shaped, anaerobic, spore-forming pathogenic bacteria and produce enterotoxins that cause diarrhea in human. Detected in the whole world, *C. perfringes* type A cause foodborne infection when, at least, 10^8 bacteria reach the intestine and produce the enterotoxin. The infection can be caught by beef and chicken cooked slowly and storage in ambient temperature. The spores resist the baking process, germinate in the food and, if the amount of bacteria is high, a significant part survive the stomach acid. The most common symptoms are watery diarrhea and intestinal spasms[10].

In Brazilian legislation, RDC n.12 by ANVISA resolution, the microbiological parameter for

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hot sandwiches and other snacks permit up to 10^2/g of thermotolerant coliforms, up to 10^3/g Coagulase-positive Staphylococci and Sulfite-reducing Clostridia, in the absence of *Salmonella* spp in 25g of food.

Therefore, the objective was to evaluate the microbiological parameters of kebab samples marketed in downtown of São Paulo, noticing if they are according to the ANVISA (RDC n.12)[12] parameters, with *Salmonella* spp detection, to determine the Most Probable Number (MPN) of thermotolerant coliforms (TtC), coagulase-positive staphylococci (CPS) enumeration and *S. aureus* identification, sulfite-reducing *Clostridium* (SRC) enumeration and research of endotoxins-producer genes in *S. aureus* strains.

2 MATERIALS AND METHODS

2.1 SAMPLES COLLECTION

100 samples of kebab, composed by meat slices, vinaigrette and bread, were collected from March 2017 till July 2017 from snack bars located in downtown Sao Paulo. The samples were kept cool, in isothermal box with recyclable ice until the analysis in the laboratory.

2.2 MICROBIOLOGICAL ANALYSIS

2.2.1 Samples preparation and dilutions

25g of sample were weighted and homogenized with 225ml of Buffered Peptone Water (BPW), in appropriate plastic bag into Stomacher Lab Blender 400 for 30 seconds. From this initial dilution of 10^{-1}, a series of decimal dilutions was prepared, using saline solution.

2.2.2 Thermotolerant coliforms Most Probable Number (MPN)[13]

1 mL of each dilution was inoculated in each series of 3 tubes dilution, containing 10 mL of Lauryl Sulfate Broth with an invert Durham tube. The tubes were incubated at 35°C for 24 to 48 hours. Positives tubes had gas production on Durham tube. Then, with an inoculation loop, an aliquot was passed three times to 5 mL *Escherichia coli* (EC) Broth tube containing an invert Durham tube, incubated at 45°C for 24 hours to TtC confirmation. After the incubation period, the reading was based on gas production in Durham tube. Next, using the MPN table, the TtC MPN was calculated per gram of sample.

2.2.3 Coagulase-positive staphylococci enumeration[14]

Serial dilutions were plated using the surface inoculation method in Baird-Parker agar supplemented with egg yolk and tellurite, the plates were incubated at 35°C for 48 hours. After the incubation time, the plates with 25 to 250 CFU (Colony-Forming Unit) were counted. Characteristics colonies were passed to an inclined TSA tube and incubated at 35°C for 24 hours. Next, the catalase

production, Gram staining method, coagulase and termonuclease tests were realized.

For *Staphylococcus aureus* identification, the Staphytect Test Dry Spot kit was used and the positive strains passed through the VP (Voges Proskauer) test, which *S. aureus* is positive and *S. intermedius* is negative.

2.2.4 *Salmonella* spp. detection[15]

For *Salmonella* spp. detection, 25 g of the sample was weighed and homogenized in a Stomacher with 225 mL of buffered peptone water (BPW) for 2 minutes and incubated at 35°C for 24 hours. After the incubation period, 1 mL was pipette into a Tetrathionate broth (supplemented with 0.2 mL of potassium iodide) and incubated at 35°C for 24 hours, and 0.1 mL was pipetted into a Rappaport-Vassiliadis broth and incubated at 42°C for 24 hours. Then, with an inoculation loop, an aliquot of these broths was plated on XLD (Xylose Lysine Deoxycholate) and SS (*Salmonella-Shigella*) agars. After the incubation at 35ºC for 24 hours, isolated characteristics colonies were passed to an inclined TSA tube, incubated at 35°C for 24 hours, then were biochemically tested using Triple Sugar Iron (TSI) and Phenylalanine agars, incubated at 35°C for 18-24 hours. The strains with typical biochemical profile were submitted to the API 20E (BioMérieux), which has 20 biochemical tests for Enterobacterium. The positive strains in API20E were tested to anti-flagellar and anti-somatic polyvalent serums (Probac).

2.2.5 Sulfite-reducing *Clostridium* (SRC) enumeration

A 0.1 mL inoculum of each dilution was plated on SPS agar (Sulfite Polymyxin Sulfadizine), after the inoculum total absorption, 8 mL of molten SPS was plated. The plates were incubated in anaerobiosis jar with anaerobic generator envelope at 43°C for 48 hours. Plates with 25 to 250 black colonies were counted. The result was calculated by CFU/g.

2.3 PCR

2.3.1 DNA extraction and identification of *S. aureus* strains

"MiniSpin" (GE Healtcare) kit was used for DNA extraction and purification, according to supplier instructions. The sample was frozen at -20°C until the moment of the analysis.

A volume of 25µL was used for PCR reaction, composed of 2.5µL of PCR Buffer 10x, 2.0 µM of magnesium chloride, 200 µM of each dNTP, 1 U of Taq DNA Polimerase, 10 picomoles of each *primer* (Table 1), ultrapure water autoclaved (qsp) (Milli-Q Plus, Millipore) and 3 µL of DNA sample. The incubation was conducted in thermal cycler PTC-100 (MJ Research, Inc.), using the parameters of initial cycle at 94°C during 5 minutes for initial denaturation, 94°C for 2 minutes for denaturation, and 72°C during 1 minute for gene of classic enterotoxins' extension (SEA, SEB, SEC e SED). For nuc (EU) gene, the parameters were an initial cycle of 94°C during 5 minutes for initial

denaturation, 94°C during 30 seconds for denaturation and 72°C during 1 minute for extension. The relation between annealing temperature of *primers* and each gene is shown in Table 1. In all reactions, a negative control was used, replacing the acid nucleic by ultrapure water.

The positive controls used for enterotoxins were *S. aureus* ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 19095 (SEC), ATCC 23235 (SED). The products of PCR reaction were submitted to electrophoresis (Electrophoresis Power Supply Model EPD 600 – Amersham-Pharmacia Biotech Inc.) in agarose gel 1.5% (Sigma Aldrich) in boric acid-Tris-EDTA (TBE) buffer and revealed with SYBR Green (Invitrogen). The DNA fragments were analyzed in comparison with DNA markers of 100bp (100 Base Pair Ladder – Amersham – Pharmacia Biotech Inc.) in image analyzer (Alphaimager

– Alpha esasy FC Software – AlphaInotech Corporation).

Table 1. Oligonucleotides and their properties used in genes producers of enterotoxins detection and *S. Aureus*

Gene	Sequence	Product length (bp)	Annealing Temperature	References
Nuc	tcagcaaatgcatcacaaacag cgtaaatgcacttgcttcagg ttggaaacggttaaaacgaa	255	57°C	Poulsen et. al (2003)
Sea	gaaccttcccatcaaaaaca tggtcaaatttatctcctggtgcaggc	120	50°C	Johnson et al. (1991)
Seb	tttaacaactcgccttatgaaacggga	101	50°C	This paper*
Sec	aattgtgtttcttttattttcataa tgtatgtatggaggaataac ctagtttggtaatatctcct	102	50°C	This paper* Johnson et al.
Sed	taatgctatatcttataggg	317	50°C	(1991)

bp: base-pair *The *primers* of genes *seb* e *sec* were designed using the program Primer Blast (http://www.ncbi.nlm.nih.gov/nuccore)

3 RESULTS

Hundred samples were collected and analyzed for *Salmonella* spp., MPN of thermotolerant coliforms (TtC), coagulase-positive staphylococci (CPS) enumeration and sulfite-reducing *Clostridium* (SRC) enumeration.

According to Brazilian legislation, RDC n°12 resolution from ANVISA (2001)[12], the microbiological parameters for this food allow until 10^2/g of thermotolerant coliforms, until 10^3/g of coagulase-positive staphylococci and sulfite-reducing *Clostridium*, in the absence of *Salmonella* spp., in 25 grams of the product.

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Among the 100 samples, 70 (70%) presented MPN of TtC in higher quantities allowed by ANVISA(2001). Among them, 36 (36%) presented more than 10^6 MPN of TtC/g, which indicates

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the beginning of food deterioration[17]. SRC was detected in 12 samples, but only 4 (4%) were out of the permitted parameters. Five samples were positive for CPS, but all within the allowed parameters. Only one sample (1%) presented *Salmonella* spp. 71 (71%) of the samples were out of the allowed parameters, since the samples with SRC also exceeded the TtC.

Among the 5 positive samples for CPS, only 3 were positive for nuc gene in PCR (Figure 1), confirming *Staphylococcus aureus* species. None of the strains presented genes for SEA, SEB, SEC or SED enterotoxins.

The snack bars 1, 2, 9 and 10 presented the higher quantitie of samples unfit for consumption (80%) and the snakbar 6 presented the lowest quantitie of contaminated samples (50%), according to Table 2.

Table 2. Characteristics of samples according to each snackbar

Snack bar	1	2	3	4	5	6	7	8	9	10
Number of collected samples	10	10	10	10	10	10	10	10	10	10
Number of samples within the parameters	2	2	4	3	4	5	3	3	2	2
% of samples out of the parameters	**80**	**80**	**60**	**70**	**60**	**50**	**70**	**70**	**80**	**80**

In relation to hygienic aspects, all the snack bars presented in the same manner. The place where the meat was cooked was placed in the sidewalk in front of the snack bar, the vinaigrette was stored without refrigeration, in a drawer under the system where the meat was cooked. The employee wore a plastic glove in the hand that manipulated the bread and was the same that manipulated the money.

4 DISCUSSION AND CONCLUSION

The street foods are very popular in big cities, since they have fast preparation and are cheap. But, Brazilian researches pointed out a high contamination in street foods by bacteria that are able to cause foodborne diseases, as *Staphylococcus aureus, Clostridium perfringens, Escherichia coli* e *Salmonella* spp.[18,19]. Our results found considerable level of contamination in the samples, showing that kebab can be a risk for consumers health. The presence of pathogens in kebab have been reported over the time. Kayisoglu et al[6] analyzed 60 kebab samples (raw and cooked, chicken and beef) collected from Tekirdag Market in Turkish notice that 40% of cooked beef samples had Salmonella spp. and Clostridium perfringens. In another study with 40 cooked kebab samples collected from 8 different restaurants in Erzurum city in Armenia, 60% of the samples presented *S. aureus* and 67,5% presented E. coli. C. perfringens was below the detection levels in 85% of the samples and Salmonella

spp. was not found7. In 2005, Elmali et al8 reported E. coli in 54% of the 100 samples and Salmonella spp. in 14% of the samples.

In Australia, 236 kebab samples were analyzed and 88,6% were according to legal standards, 11 samples presented high levels of E. coli, 2 had high level of C. perfringens and Salmonella spp. was not detected [9].

We believe the reason of kebab present high level of contamination independent of country is due to preparation mode besides of the hygienic and sanitary conditions of utensils, equipments and kebab handlers. In all 10 snack bars, which the samples were collected, the food handlers also handled the money, the vinaigrette was stored without refrigeration, in a drawer under the system where the meat was cooked and next to the money's drawer, the handler wore a plastic glove only in one hand, the hair wasn't protected, the spoon used to take the meat was the same to take the vinaigrette and the kebab equipment was placed in the sidewalk, subjected to dirt brought by the wind and contamination by insects.

The results about this study show uniformity with previous studies about street food, pointing out the similarity of handling, preparation and conservation of these kind of food, within the results of microbiologial analysis, it was shown that kebab is a risk for consumers health, since the majority of the samples was out of the limits allowed by ANVISA.

Therefore, its necessary to create standard operating procedure (SOP) and to intensify the inspection by official responsible, so that handlers can have an standard to storage and prepare, improving hygienic and sanitary conditions.

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