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In vitro Microbial Susceptibility to Celery Acetic Extract Powder and the Sensory Impact When Applied to Fresh Chicken Sausage

Gisely de Mattos[a], Leila Larisa Medeiros Marques[a]*, Flávia A. R. Cardoso[a], André F. Guerra[b]

[a] Universidade Tecnológica Federal do Paraná, Paraná, Brasil
[b] Centro Federal de Educação Tecnológica Celso Suckow da Fonseca, Rio de Janeiro, Brasil
leilamarques@professores.utfpr.edu.br

As a result of the negative aspects of the consumption of synthetic food additives, consumers have shown a strong inclination to consume preparations with natural ingredients. The purpose of this work was to evaluate the in vitro microbial susceptibility of powdered celery acetic extract (PCAE) on autochthonous and allochthonous microorganisms in fresh chicken sausage; produce fresh chicken sausage on a pilot-industrial scale and evaluate the sensory impact of adding PCAE; and characterize the physical-chemical parameters. In vitro microbial susceptibility was evaluated by the turbidimetric method. In the in-situ test, five sausage formulations were carried out. The in vitro assay revealed that *Staphylococcus aureus* and *Clostridium perfringens* were resistant up to 5.0% PCAE. Partial susceptibility was detected at concentrations of 2.5 to 5.0% PCAE for *Pseudomonas aeruginosa*, *Salmonella* serovar Typhimurium, *Listeria innocua* and *Escherichia coli*. None of the microorganisms tested were susceptible to PCAE. All sausages complied with the physical-chemical standards, established by Brazilian legislation, but the values of residual nitrite, in T1, T2 and T3, exceeded the prescribed upper limit. The addition of 2.5%, and 3.5% of PCAE did not negatively influence the sensory attributes of the sausage. It is concluded that the PCAE is capable of partially inhibiting some microorganisms that commonly occur in sausages without negatively interfering in the sensorial attributes, contributing to the microbiological safety.

1. Introduction

During the production process, fresh preparations are not subjected to heat treatment. Therefore, there is no reduction in the pre-established autochthonous and allochthonous microbial load and because it has intrinsic factors favorable to microbial growth, the commercial validity (shelf-life) is generally short (Souza, 2014).
Deteriorating and pathogenic microorganisms can be transmitted by chicken meat and derivatives. A series of factors can contribute to the contamination of the product, such as: raw material, handlers, utensils, hygiene of the processing environment, among others. Additives, which inhibit microbial growth, are used to impart microbiological safety to the product (Idris and Nadzir, 2017). The use of curing salt such as nitrite and/or nitrate and lactate are additives that control microbial growth in meat products (Adami, 2015). The use of sodium nitrite in meat sausages is the most effective method to control the clostridial group, including *C. botulinum and C. perfringens*. Faced with the current rejection of the consumption of synthetic additives, consumers have avoided the consumption of foods with direct addition of nitrite/nitrate. An imminent risk arises from the presence of toxigenic clostridia. Therefore, studies are being carried out to develop natural alternatives with a paired effect on nitrite and lactate (Lee et al., 2019).

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2. Methodology

2.1 In vitro microbial susceptibility

The turbidimetric method was used to assess the susceptibility of *Salmonella* serovar *Typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Listeria innocua* (ATCC 33090), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Clostridium perfringens* (ATCC 13124) to the PCAE. The cultures were activated (3x) in brain heart infusion broth - BHI and incubated at 36 °C. A hermetically closed jar with an anaerobic generator sachet was used for activation of *C. perfringens* under anaerobiosis. Cellular biomass was obtained by centrifugation at 6000g for 5 minutes and double washing of the pellet with phosphate buffer pH 7.2. The biomass remaining after the last separation was reconstituted in BHI broth until obtaining a cell suspension equivalent to 5.5 log CFU/mL. Calibration curves were previously plotted and used to help adjust the inoculum. PCAE, purchased commercially, was diluted in BHI broth to obtain a concentration of 0.5 to 5.0%, with increments of 0.5% from tube to tube. Tubes devoid of mixture and inoculum were included as control and assay blank, respectively. The tubes were incubated at 36 °C in a thermostatic bath with agitation and the turbidity was determined by measuring the absorbance at 600λ in a spectrophotometer at times 0, 13, 16, 19, 22, and 37 hours. *C. perfringens* was incubated under anaerobiosis and incubated in a B.O.D. During the analytical series, the white tube was used to calibrate the equipment, the external surface of the tubes was dried with a paper towel and the absorbance value was measured directly in the tubes, dispensing with the use of a cuvette. The potential dose to achieve an inhibitory effect on each strain was expressed considering three categories: i) susceptible, statistically ($p < 0.05$) absorbance value equal to the blank; ii) partially susceptible, absorbance value between the white tube and the control; iii) resistant, absorbance value statistically ($p < 0.05$) equal to the control tube.

2.2 Production of fresh chicken sausage

Five formulations were produced, being white, control and three treatments with PCAE doses that showed some inhibitory effect in the in vitro assay. White (B): without curing salt, sodium lactate and PCAE; Control (C): with 0.15% curing salt and 2.0% sodium lactate; Treatment 1 (T1): with 0.15% curing salt and 2.5% PCAE; Treatment 2 (T2): with 0.15% curing salt and 3.5% PCAE; Treatment 3 (T3): with 4.5% PCAE. The formulations are described in detail in Table 1.

Table 1: Fresh chicken sausage formulations without or with PCAE (powdered celery acetic extract).

	Treatments				
Ingredients	B	C	T1	T2	T3
Chicken meat	86.81	86.81	86.81	86.81	86.81
Soy protein	2.5	2.5	2.5	2.5	2.5
Potable water	8	8	8	8	8
Salt (sodium chloride)	1.2	1.06	1.06	1.06	1.2
Condiment (salt, sugar, spices and aroma)	1.04	1.04	1.04	1.04	1.04
Sodium tripolyphosphate	0.35	0.35	0.35	0.35	0.35
Sodium erythorbate	0.1	0.1	0.1	0.1	0.1
Curing salt (salt and sodium nitrite)	0	0.15	0.15	0.15	0
Sodium lactate	0	2	0	0	0
PCAE	0	0	2.5	3.5	4.5

2.3 Sensory evaluation

A panel of 60 tasters, untrained, composed of 15 men and 45 women, between 22 and 50 years old, declared as sausage consumers, was randomly selected. The sausages were prepared on an electric grill for 30 minutes. After roasting, they were cut into portions of approximately 20 grams and served to tasters according to each test. The sensory attributes 'identification of the standard sample in relation to the treatment samples' were quantified by the difference test (duo-trio). Panel members received a standard sample (P) and two samples coded with random numbers (three digits), and each panelist was asked to identify the treatment sample with attributes like the standard. Concomitantly, an acceptance test was also carried out, using a hedonic scale with a 9-point score. Finally, tasters were asked to identify the preferred sample among the samples added with PCAE.

2.4 Statistical evaluation of data

Linear regression was applied to the results of the turbidimetric method. The angular coefficients of the regressions (mean ± standard error) were evaluated by analysis of variance (ANOVA) followed by the non-parametric Kruskal-Wallis test ($p < 0.05$). Physicochemical analyzes were performed in triplicate. The results were evaluated by analysis of variance (ANOVA) using the Tukey test at the 5% level of significance with the aid of the BioEstat 5.3 program.

3. Results and discussion

3.1 In vitro microbial susceptibility

The in vitro microbial susceptibility results are shown in Tables 2, 3, and 4.

Table 2: Linear regression parameters of microbial growth of Salmonella serovar Typhimurium and Escherichia coli.

Microorganism		*Salmonella* Typhimurium				*Escherichia coli*			
Coefficients		xi	yi	R^2	SE	xi	yi	R^2	SE
Concentration (%) of PCAE	0	0.0709^{ab}	0.0638	0.9613	3.8058	0.0776^{a}	0.1140	0.9225	2.7322
	0.5	0.0729^{a}	0.1333	0.9367	2.5656	0.0787^{a}	0.1047	0.9372	2.8964
	1	0.0727^{a}	0.0746	0.9450	3.4599	0.0700^{ab}	0.1128	0.8867	2.6401
	1.5	0.0701^{abc}	0.1196	0.9371	2.7097	0.7410^{a}	0.1083	0.9277	2.8190
	2	0.0710$^{a}_{b}$	0.1045	0.9225	2.8537	0.0666^{abc}	0.0730	0.9136	3.3814
	2.5	0.0648^{abc}	0.1076	0.9144	2.7876	0.0635^{abc}	0.0669	0.9387	3.6292
	3	0.0627^{bc}	0.1205	0.9352	2.6941	0.0606^{abc}	0.0588	0.9046	3.7305
	3.5	0.0626^{abc}	0.0891	0.9144	3.0634	0.0550^{abc}	0.0593	0.8818	3.6211
	4	0.0607^{c}	0.0552	0.9446	4.0205	0.0474^{bc}	0.0385	0.8856	4.5134
	4.5	0.0564^{bc}	0.0678	0.9099	3.4944	0.0377^{bc}	0.0659	0.8911	3.4712
	5	0.0481^{c}	0.0997	0.7781	2.4643	0.0331^{c}	0.0283	0.8804	5.2334

*Number of observations (8); degrees of freedom (6); Student's t-value (2.9687); confidence level (0.95). Different lowercase letters indicate significant differences by the Kruskal-Wallis test ($p<0.05$).

Table 3: Linear regression parameters of the microbial growth of Listeria innocua and Pseudomonas aeruginosa.

Microorganism		*Listeria innocua*				*Pseudomonas aeruginosa*			
Coefficients		xi	yi	R^2	SE	xi	yi	R^2	SE
Concentration (%) of PCAE	0	0.0496^{a}	0.1533	0.9205	2.3510	0.0708^{a}	0.1010	0.9138	2.8753
	0.5	0.0340^{ab}	0.0503	0.9583	4.2728	0.0721^{a}	0.0602	0.9246	3.7684
	1	0.0270^{abc}	0.0675	0.9470	3.6450	0.0643^{ab}	0.1519	0.9221	2.3659
	1.5	0.0149^{abcd}	0.0928	0.8695	2.8543	0.0633^{ab}	0.1310	0.9207	2.5438
	2	0.0148^{abcd}	0.1000	0.8288	2.6209	0.0610^{ab}	0.1287	0.9262	2.5818
	2.5	0.0126^{abcd}	0.0742	0.7692	2.8238	0.0584^{bc}	0.1579	0.9116	2.2941
	3	0.0091^{d}	0.1612	0.6605	1.6451	0.0572^{bc}	0.1441	0.9320	2.4552
	3.5	0.0112^{bcd}	0.0997	0.5860	1.8559	0.0552^{bc}	0.1259	0.9339	2.6320
	4	0.0156^{abcd}	0.0479	0.8673	3.9628	0.0447^{c}	0.1316	0.9645	2.6587
	4.5	0.0122^{abcd}	0.1188	0.6632	1.9241	0.0409^{c}	0.1404	0.8930	2.3832
	5	0.0091^{cd}	0.1326	0.5197	1.4272	0.0314^{c}	0.1404	0.9163	2.4454

*Number of observations (8); degrees of freedom (6); Student's t-value (2.9687); confidence level (0.95). Different lowercase letters indicate significant differences by the Kruskal-Wallis test ($p<0.05$).

Table 4: Linear regression parameters of microbial growth of Staphylococcus aureus and Clostridium perfringens.

Microorganism		*Staphylococcus aureus*				*Clostridium perfringens*			
Coefficients		xi	yi	R^2	SE	xi	yi	R^2	SE
Concentration (%) of PCAE	0	0.0721[a]	0.0919	0.9202	3.0355	0.0580[a]	0.1203	0.9100	2.6237
	0.5	0.0726[a]	0.1043	0.9491	2.9388	0.0592[a]	0.1000	0.9081	2.8717
	1	0.0731[a]	0.1377	0.9334	2.5154	0.0694[a]	0.1107	0.9290	2.7922
	1.5	0.0700[a]	0.1273	0.9378	2.6284	0.7050[a]	0.1052	0.9385	2.8935
	2	0.0676[a]	0.1479	0.9362	2.4344	0.0742[a]	0.0945	0.9313	3.0295
	2.5	0.0662[a]	0.1251	0.9383	2.6529	0.0742[a]	0.1125	0.9445	2.8160
	3	0.0637[a]	0.1514	0.9329	2.3976	0.0736[a]	0.1269	0.9146	2.5674
	3.5	0.0638[a]	0.1721	0.9405	2.2671	0.0708[a]	0.0977	0.9539	3.0518
	4	0.0644[a]	0.1314	0.9467	2.6116	0.0665[a]	0.1161	0.9497	2.7872
	4.5	0.0598[a]	0.1824	0.9404	2.2019	0.0624[a]	0.0927	0.9642	3.1668
	5	0.0582[a]	0.1857	0.9088	2.1089	0.0599[a]	0.0776	0.9790	3.5144

*Number of observations (8); degrees of freedom (6); Student's t-value (2.9687); confidence level (0.95). Different lowercase letters indicate significant differences by the Kruskal-Wallis test (p<0.05).

Staphylococcus aureus and *Clostridium perfringens* were resistant up to 5.0% PCAE. Partial susceptibility was detected at concentrations between 2.5 and 5.0% of PCAE for *Pseudomonas aeruginosa*, between 3.0 and 5.0% for *Salmonella* Typhimurium and *Listeria innocua*; and between 4.0 and 5.0% for *Escherichia coli.* None of the microorganisms tested showed full susceptibility to PCAE. *P.* aeruginosa is a microorganism commonly associated with the deterioration of a variety of foods, including meat and meat products, since it has activity under aerobic conditions and under refrigeration temperatures (Benvenutti, 2020).

3.2 Physicochemical analysis

There was no significant difference ($p < 0.05$) between samples regarding protein and calcium contents. All samples showed significant differences in fat content. Samples C and T1 were the samples with the highest and lowest fat content, respectively. For moisture, only samples C and T3 did not differ significantly from each other. Sample T1 was the one with the highest moisture. Differences in fat and moisture values are inherent to the formulations, due to ingredient variation. All samples showed values of moisture, protein, fat and calcium content on a dry basis, in accordance with the standards established by Normative Instruction No. 4, of March 31, 2000, of the Ministry of Agriculture, Livestock and Food Supply (Brasil, 2000). The pH values varied little and only samples B and T3 showed significant differences between their means. Acidic pH interferes with the curing reaction, shifting the chemical balance in favor of nitric oxide formation. Consequently, reducing the residual nitrite content. Nitric oxide and other secondary products of the nitrite reaction have antimicrobial action (Horsch et al., 2014). The results of the physical-chemical analyzes are presented in Table 5.

Table 5: Physical-chemical analyzes for fresh sausage samples (mean ± standard deviation).

	Samples				
Parameters	B	C	T1	T2	T3
Moisture (%)	62.47[b]±0.07	60.49[d]±0.03	62.95[a]±0.09	61.68[c]±0.04	60.36[d]±0.01
Protein (%)	14.32[a]±0.04	13.92[a]±0.05	15.51[a]±0.08	15.41[a]±0.05	16.72[a]±1.50
Fat (%)	18.48[c]±0.02	20.61[a]±0.03	16.67[e]±0.01	17.83[d]±0.10	19.71[b]±0.02
pH	6.48[b]±0.00	6.53[ab]±0.02	6.69[ab]±0.10	6.61[ab]±0.01	6.72[a]±0.01
Nitrate (ppm)	0.42[d]±0.36	54.74[c]±0.35	134.92[b]±2.38	165.06[a]±0.09	167.75[a]±5.81
Nitrite (ppm)	0.00[e]±0.00	84.28[d]±0.60	261.71[c]±0.66	324.85[a]±5.78	303.90[b]±0.26
Calcium content on a dry matter basis (%)	0.1	0.1	0.1	0.1	0.1

* Different lowercase letters indicate significant differences by Tukey's test (p<0.05).

Regarding the values of nitrate and nitrite, T2 and T3 samples differed significantly ($p < 0.05$). The highest values of nitrate and nitrite were observed in sample T2 and the lowest in sample B. Samples T1, T2 and T3 had values above the 150 ppm of nitrite allowed by Brazilian legislation. Synthetic nitrite was maintained in the formulation of samples T1 and T2, as PCAE did not demonstrate an inhibitory effect on *Clostridium perfringens* in the in vitro assay. In the study carried out by Myers et al. (2013), using synthetic nitrite and celery powder, it was reported that nitrite values, when coming from a natural source, are more stable and decrease at a slower kinetics compared to synthetic nitrite.

3.3 Sensory analysis

In the duo-trio test, 65% of the tasters identified the T1 sample different from the standard and 86.67% of the tasters identified a difference between the T2 sample and the standard. The T1 and T2 formulations did not present a significant difference in relation to the mean scores in the acceptance test and in the preference between them. The tasters noticed a significant difference between the samples without and with the addition of PCAE, however, given the acceptance test scores, they did not reject the samples with 2.5% and 3.5% of PCAE. This result confirms the results found in a previous study by Jin et al. (2018), in which the addition of celery did not generate any unpleasant taste in meat products. The presence of vinegar in the percentages of the mixture evaluated also did not bring negative sensory attributes, as concentrations of 2% or 2.5% can cause changes in the sensory characteristics of the products (Ponrajan et al., 2012).

4. Conclusions

PCAE is capable of partially inhibiting some microorganisms that commonly occur in fresh chicken sausage, such as *P. aeruginosa*, *Salmonella sp*. and *E. coli*, contributing to the microbiological safety of the matrix. However, the concomitant use of PCAE and the direct addition of sodium nitrite, at the doses used in treatments T1, T2 and T3, goes beyond the maximum limit allowed by Brazilian legislation. Due to the resistance of C. perfringens to PCAE, the total replacement of sodium nitrite by pre-converted celery nitrite must be analyzed with caution. This highlights the importance of advancing studies regarding the inhibitory potential of natural sources to achieve the same level of microbiological safety as synthetic nitrite, especially in relation to the clostridial group.

Nomenclature

xi - angular coefficient
yi - linear coefficient
R2 - coefficient of determination
SE - standard error

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