Chapter 67

Antimicrobial potential of *Hansenula wingei* dry extract in poultry cuts

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1 INTRODUCTION

As poultry slaughter volume increases in Brazil, which ranks third in the world in production (EMBRAPA, 2020), this industry has developed new technologies to improve the quality of cuts. Brazilian poultry is internationally recognized and one of the most popular worldwide. Its versatility in properly meeting the world's demands has afforded the country world leadership in exports. However, restrictions due to the presence of pathogenic microorganisms (*Salmonella* spp.) have had a significant impact on animal production and especially on food safety. As it is highly perishable, just like other meat products, improper slaughtering, conservation, and transport operations can affect poultry quality, impairing its acceptance and trade. Thus, preserving product characteristics and prolonging its shelf life have been major challenges for the poultry industry.

Until the final product is obtained, cuts and carcasses are susceptible to high levels of contamination, since, in addition to demanding time, the production system itself contributes to an increase in microbial loads. Moreover, there are issues intrinsic to the quality of raw materials and process hygienic conditions, influencing contamination levels of the final product.

Several factors within the industry can lead to poor quality of the final products, such as non-compliance with good manufacturing practices, lack of preventive maintenance, raw material uniformity, and operator training. These are critical aspects that can lead to non-compliance with operational sanitary procedures (PSO, in Portuguese), with consequent contamination of chicken carcasses, thus promoting colonization of bacteria such as *Salmonella* spp. Such contaminations can originate from live birds or be incorporated during the process, such as gastrointestinal disruption during evisceration when PSO requirements are not met. These contaminants must be controlled and monitored due to food safety, as well as for a better quality of the meat according to the microbiological standards established by Normative Instruction n. 60, of December 23, 2019, of the Ministry of Health and the National Health Surveillance Agency (ANVISA, in Portuguese).

Currently, antimicrobials have been used in several process stages of the meat industry; during pre-evisceration carcass washing, equipment spraying during sanitization, evisceration, cutting, post-evisceration internal and external washing of carcasses, carcass cooling, and post-cooling. The most commonly used are peracetic acid (PAA), cetylpyridinium chloride (CPC), acidified sodium chlorite (ASC), organic acids, bromine, and chlorine, following all Good Manufacturing Practices (GMP), and control of critical points (CPs), such as temperature during cooling at 4 °C for up to 4 hours.

The goal of this study consisted of using a natural antimicrobial alternative with the same control effect, the dry extract of *Hansenula wingei* yeast, to improve the microbiological quality of poultry cuts and carcasses, as it is a sensitive product physically, chemically, and microbiologically.

2 MATERIAL AND METHODS

Cuts and carcasses were purchased from a slaughterhouse of the company Seara, which is in the city of Rolândia, Paraná State, Brazil. Microencapsulated extract of Hansenula wingei yeast was provided by the Federal Technological University of Paraná (UTFPR), Campus of Londrina, Paraná State, Brazil. For microbiological analysis, *Salmonella* spp. strains were isolated at the laboratory of the company Seara, in Rolândia. Culture media and reagents used were acquired by the laboratory where tests took place.

Crude extract of *Hansenula wingei* yeast (Hw): From a solid culture of Hw on potato dextrose agar (PDA), a pre-inoculum was previously standardized on McFarland Scale ($3.0x10^7$ CFU/mL), and an aliquot of 100 µL was transferred to six 1-L flasks containing broth medium for yeasts (MPL Broth: 2% glucose, 0.5% yeast extract, 1% sodium chloride, 0.5% ammonium sulfate, and 1% monobasic sodium phosphate). A total of 12 L was prepared and incubated in BOD at 25 ºC for 148 hours. After incubation, the product was centrifuged at 10,000 rpm for 10 min, and the supernatant was separated from the precipitate. The supernatant was subjected to spray drying using a spray dryer as the following parameters: feed flow of 0.7L/h, the inlet temperature of 112°C, airflow 1.93m³/h, and nozzle of 0.7mm.

Minimum inhibitory concentration: To assess the efficiency of killer toxin in Hw dry extract, after concentration by drying, minimum inhibitory concentration (MIC) tests were performed. Dry extract of Hw

was diluted to a concentration of 0.25g/ mL. As a negative control, 1.5% lactic acid was used. Test bacteria were activated in BHI broth for 24 h in a microbiological growth oven at 38 °C, and then inoculated onto Mueller Hinton (MH) medium plates for 24 h for growth and adaptation to the test medium. Afterward, the colonies were diluted in MH broth at 0.5 McFarland scale, which corresponds to $1.5x10^8$ CFU/mL, and then diluted again 100x to count corresponding to $1.5x10^6$ CFU/ mL. Each well was added with 100-µL MH broth and, excluding the control, 100-µL test bacteria (1.5x105 CFU) and six aliquots of the diluted extract were added: 50, 60, 70, 80, 90, and 100 µL, thus constituting six concentrations of dry extract to be tested to find the lowest concentration to cause full inhibition of the tested microorganisms. The concentrations were: 0.041g/mL, 0.05g/mL, 0.058g/mL, 0.066g/mL, 0.075g/mL, and 0.083g/ mL.

Determination of *Salmonella* spp. in chicken breast cuts: For the initial counts in cuts and carcasses, to make sure they were not contaminated with *Salmonella* spp., quantification was performed using the equipment known as "VIDAS" using the method ELFA (Enzyme Linked Fluorescent Assay).

Determination of *Salmonella* spp. by plating: XLD agar (xylose lysine deoxycholate) was used (Koneman et al., 2008), and the experiment was carried out in two stages: a) in cuts (breast fillet) and b) in carcasses, all cooled. An initial count was performed in samples to quantify initial contamination and, later, to verify the efficiency of tests to be carried out with Hw dry extract. Samples were pre-tested for *Salmonella* spp. and then added with a known concentration of the microorganism.

Salmonella spp. inoculum preparation and sample contamination: For the beginning of tests, the initial *Salmonella* spp. concentration to be inoculated into samples was determined since samples were tested as abs/25g. An inoculum solution of 1x10-3 (MacFarland scale) *Salmonella* spp. was prepared from an XLD plate. This material was incubated in an oven at 35 ºC for 24 h +/- 3 h, obtaining cultures to contaminate the meat samples. After contamination (breast fillet and carcass samples), *Salmonella* spp. counting was performed at 0 and 14 days, with samples being kept in sterile bags and refrigerated at temperatures between 0 and 4º C. Two experiments were carried out, one on carcass and another on breast fillet.

Following is a description of the experiments performed: - Experiment 1: 18 g Hw extract was diluted in 600 mL sterile water and divided into 6 equal parts, half of which were destined to breast fillet cut and the other half to half carcass. Then, 100 mL dry extract solution was prepared and added to the samples. - Experiment 2: We only used a solution with $1x10^{-3}$ *Salmonella* spp. added to 100 mL sterile water. - Experiment 3: 1.5 mL peracetic acid (pa; 1% concentration) was added with a $1x10^{-3}$ *Salmonella* spp. concentration. For all experiments, the procedure was the same for fillets and half carcasses, and experiments were carried out in triplicates.

All experimental samples were kept in sterile bags and refrigerated at temperatures between 0 and 4º C. After 24 h and 14 days for breasts, and 24 hours and 4 days for carcasses, the following protocol was followed: a portion of 25 g was removed, hydrated with 225 mL 1% peptone water, and homogenized, inoculating 1 mL on an XLD plate and spread with a Drigalski loop and incubated for 18h/24h at 43ºC.

Data were analyzed by the BioStat software, using the student's t-test to verify differences between treatments.

3 RESULTS AND DISCUSSION

Extracts yielded on average 0.66% over crude extract. A total of 12 L fermented Hw was prepared and, after drying, 8 g dry extract was obtained. The drying method should be further explored, with potential microencapsulation to optimize yield and facilitate the process, since yeast drying is relatively time-consuming and wastes a large amount of caramelized material inside the equipment. This was the first test to obtain dry extract for application in meat samples. From this extract, the results obtained are discussed below.

To test turbidity, we used 96-well plates, where the less turbid, the greater inhibition of tested bacteria. The use of extract inhibited pathogenic strains (*Salmonella* spp.) at the highest concentration tested (0.083 g/mL), while some strains were inhibited at lower concentrations, such as *E. coli* and coagulase-positive *Staphylococcus*. The MIC defined from these observations was 0.083 g/mL, i.e., the highest concentration tested. Initial analyses were performed with 25 g of each sample. For breast fillets, results of 103 were observed, while for carcasses results differed from what was inoculated. This may have occurred due to the lack of mechanical homogenization, since, given the size, the carcass was manually homogenized, while for the fillet we used a stomacher.

Table 1 shows the results obtained for chicken breast, with initial and final counts of the microorganisms under study made in triplicates. Based on this, we observed that *Salmonella* spp. counts decreased by 1 log, which was already predicted since acetic acid is an efficient antimicrobial for the inhibition of microorganisms, therefore used as a control.

Table 1 – CFU/g counts of *Salmonella* spp. and acetic acid incubation in chicken breast samples on days 1 and 15 of refrigerated storage.

Day 1	**Chicken breast + *Salmonella* spp. + 1% acetic acid solution**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. per 25 g[1]	$2x10^3$	$4x10^3$	$10x10^3$
	$2x10^3$	$2x10^3$	$2x10^3$
	$1x10^3$	$3x10^3$	$16x10^3$
Day 15	**Chicken breast + *Salmonella* spp. + 1% acetic acid solution**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. per 25g[2]	$21x10^2$	$31x10^2$	$53x10^2$
	$15x10^2$	$25x10^2$	$12x10^2$
	$21x10^2$	$26x10^2$	$30x10^2$

1 – CFU/g count on day 1 of inoculation
2 – CFU/g count on day 15 of inoculation

Treatments with only the presence of *Salmonella* spp. (Table 2) were analyzed by Student's T-test (independent samples) and had p-value below 0.05, thereby, final and initial CFU/g counts were different. Accordingly, refrigeration was an obstacle to the growth of the microorganism, but it continued to be present in significant amounts in samples.

Table 2 – CFU counts/g of *Salmonella* spp. incubation in chicken breast samples on days 1 and 15 of refrigerated storage without the addition of antimicrobial agents.

Day 1	**Chicken breast + *Salmonella* spp.**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. / 25g[1]	$2x10^3$	$4x10^3$	$10x10^3$
	$2x10^3$	$2x10^3$	$2x10^3$
	$1x10^3$	$3x10^3$	$16x10^3$

Day 15	**Chicken breast + *Salmonella* spp.**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. / 25g[2]	$288x10^2$	$220x10^2$	$164x10^2$
	$312x10^2$	$100x10^2$	$208x10^2$
	$320x10^2$	$116x10^2$	$148x10^2$

1 – CFU/g count on day 1 of inoculation
2 – CFU/g count on day 15 of inoculation

The final test was performed after 14 days, using 25 mL of the liquid in which cuts/carcasses had contact for 14 days. The results were satisfactory from 103 to 102 for some samples, while in others it remained with the same exponential, as shown the following Tables.

Table 3 – CFU counts/g of *Salmonella* spp. incubation in chicken breast samples on days 1 and 15 of refrigerated storage with the addition of *Hansenula wingei (Hw)* dry extract

Day 1	**Chicken breast + *Salmonella* spp. + Hw extract**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. / 25g[1]	$1x10^3$	$3x10^3$	$1x10^3$
	$0x10^3$	$2x10^3$	$2x10^3$
	$1x10^3$	$2x10^3$	$1x10^3$

Day 15	**Chicken breast + *Salmonella* spp. + Hw extract**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. / 25g[2]	$3x10^2$	$47x10^2$	$86x10^2$
	$2x10^2$	$48x10^2$	$28x10^2$
	$18x10^2$	$59x10^2$	$39x10^2$

1 – CFU/g count on day 1 of inoculation
2 – CFU/g count on day 15 of inoculation

Student's t-test was applied to independent in *Salmonella* spp.-contaminated chicken breast samples. When comparing samples with and without Hw dry extract addition, there was a significant difference of 5%. Therefore, the addition of dry extract reduces the growth of this microorganism (Table 4).

Table 4 – Comparison of results from day 15 of refrigerated storage of chicken breast with and without *Hansenula wingei* (*Hw*) dry extract

Day 15	**Chicken breast + *Salmonella* spp.**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. / 25g[1]	$28x10^2$	$22x10^2$	$16x10^2$
	$3x10^2$	$10x10^2$	$20x10^2$
	$32x10^2$	$11x10^2$	$14x10^2$

Day 15	**Chicken breast + *Salmonella* spp. + Hw extract**		
	Sample 01	Sample 02	Sample 03
Salmonella spp. / 25g[2]	$3x10^2$	$5x10^2$	$8x10^2$
	$2x10^2$	$5x10^2$	$3x10^2$
	$18x10^2$	$6x10^2$	$4x10^2$

1 – CFU/g count on day 1 of inoculation
2 – CFU/g count on day 15 of inoculation

Our study stands out from the others in the literature in terms of *Salmonella* spp. control efficiency. For example, Testa et al. (2020) studied the natural antimicrobials niacin and polylysine in beef but did not have positive results against *Salmonella* spp.

Likewise, Zapata-Álvarez et al. (2019) used rosemary extract in sausages and bologna and obtained positive results against *E. coli* and *L. monocytogenes*, but did not control *Salmonella typhimurium*. The authors suggested incorporating other adjuvants such as sodium lactate (2.5%) and sodium diacetate (0.2%) for an efficient *Salmonella* spp. control. On the other hand, Radic et al. (2018) cited that basil and rosemary contain antimicrobial properties that act on gram-negative bacteria such as *Salmonella* spp., specifically for poultry meat. Other authors work with pomegranate juice to prolong the shelf life of chicken breast, obtaining satisfactory results for *Pseudomonas* spp., lactic acid bacteria, *Enterobacteriaceae*, psychrotrophic bacteria and yeasts, thus increasing the shelf life of these products by five days (BAZARGANI-GILANI et al., 2015).

Based on our results and the findings of authors who developed experiments on other natural antimicrobials, the product developed here, which is a peptide, stands out once again. According to Tiwari et al. (2009), antimicrobial peptides, or bacteriocins, are promising solutions to the problem of antibiotic resistance since they quickly destroy the microorganism's membrane, thus inhibiting a fast growth of bacteria or even their mutation in food products.

4 CONCLUSION

Our study demonstrates the antimicrobial action of Hansenula wingei dry extract; however, more repetitions should be tested for more refined conclusions. A priori, we may conclude that this yeast is a viable alternative as an antimicrobial for poultry meat. In general, we may state that: dry extract production was possible but must be optimized; the minimum inhibitory concentration of Hw dry extract on *Salmonella* spp. was 0.083g/mL; during cold storage, both breast and carcass, the number of microorganisms reduced significantly.

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