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Impact of phenolic compounds on proteolytic activity of Pseudmonas aeruginosa in UHT milk

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Psychrotrophic bacteria are commonly found in raw milk and on surfaces in the food industry. They can form biofilms and cause sensory problems in dairy products. *Pseudomonas* spp. can produce extracellular enzymes that affect milk quality, and the production of proteases at room temperature and refrigeration reinforces the need for strict microbiological control. Some studies have shown that phenolic compounds present antimicrobial activity and can inhibit phenotypes including pyocyanin production, motility, biofilm formation, and protease production which are regulated by quorum sensing in *P. aeruginosa*. The objective of this study was to evaluate the effect of phenolic compounds on the proteolytic activity of *P. aeruginosa* PAO1 (PAO1) using UHT skim milk (UHT-SM) as substrate. Four phenolic compounds (rosmarinic acid, baicalein, curcumin, and resveratrol) were evaluated at final concentrations of 50, 250, and 500 µM. Proteolytic activity in solid medium was evaluated on plates containing Luria Bertani agar supplemented with 10% UHT-SM and the phenolic compounds independently, followed by incubation (37 ºC/24 h) and observation of translucent halos around the colonies, indicating milk proteins hydrolysis. For the fluid milk spoilage test, 9 mL aliquots of UHT-SM were supplemented with the phenolic compounds independently and inoculated with PAO1 (10^5 CFU/mL), followed by incubation (25 ºC for 24 and 48 h) and evaluation of clot formation and/or color changes due to pigment production by PAO1. To evaluate the extent of milk protein hydrolysis, the SDS-PAGE method was used. A 500 µL aliquot from the fluid milk spoilage tests was mixed with sample buffer, followed by heat treatment. The samples were loaded into polyacrylamide gels (12%) and the proteins were separated by electrophoresis. Negative control (NC) was UHT-SM, and positive control (PC) was UHT-SM inoculated with PAO1 without phenolic compounds. In solid medium, a reduction in proteolysis halos was observed only in the presence of curcumin and resveratrol (500 µM), indicating partial inhibition of PAO1 proteolytic activity. In the visual assessment of fluid milk, the spoilage was observed after 48 h, with clot formation, odor change, and pigment production by PAO1. In samples containing baicalein, curcumin, and resveratrol (250 and 500 µM), spoilage intensity was lower: samples containing curcumin and baicalein had a fluid appearance similar to NC, while resveratrol reduced pigment production. In SDS-PAGE, after 24 h of incubation, a partial reduction of casein bands was observed compared to NC. After 48 h, no casein bands were observed, indicating total hydrolysis of these proteins in all treatments. Whey proteins were partially hydrolyzed, with a reduction in the intensity of bands corresponding to these proteins. In summary, the four phenolic compounds, under the tested conditions, did not effectively prevent spoilage and proteolytic activity caused by PAO1, probably due to the interaction of the compounds with the food matrix, reducing their antimicrobial and anti-quorum sensing activity. Previous studies indicated that phenolic compounds have a strong affinity for proteins, forming protein-polyphenols complexes, which also affect antioxidant activity in dairy products enriched with phenolic compounds. Further studies are needed to understand how these interactions occur in the

presence of *P. aeruginosa*, which produces many proteases.

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