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Use of phenolic compounds from cocoa pod-husks (*Theobroma cacao* L.) as inhibitors of *Salmonella* spp. in fresh cheese produced in Manabí, Ecuador

Universidad Nacional de Colombia

Uso de compuestos fenólicos del pericarpio de cacao (*Theobroma cacao* L.) como agentes inhibidores de *Salmonella* spp. en queso fresco producido en Manabí, Ecuador

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ABSTRACT

Keywords:
Cheese
Cocoa pod-husk
Fine aroma cocoa
Growth curve measurement
Phenolic compounds
Sensory evaluation

Cocoa pod-husk is a by-product of cocoa processing, underutilized despite its phenolic compounds that can be an alternative to preserve the microbiological quality of food. The aim of this work was to evaluate the *in vitro* inhibitory activity of phenolic compounds from the cocoa pod-husk against *Salmonella* spp, which is commonly found in fresh cheese produced in Manabí, Ecuador; as well as the effect on the sensory characteristics of cheese after immersion in a solution of phenolic compounds. *In vitro* microbiological analyzes of the inhibitory activity of phenolic compounds, showed that the concentrations 1 and 1.5% had the highest zone of inhibition against *Salmonella* spp., with mean diameters of 10.67 and 11.8 mm, respectively. On the other hand, the growth curve of *Salmonella* spp. indicated that 2 h were required for complete inhibition of bacteria by phenolic compounds at concentrations of 1 and 1.5%. For the sensory analyzes of cheese treated with phenolic compounds, 56.3% of the panelists accredited the firmness and odor with “I like it”, while 37.5% of the panelists qualified the color of the cheese with “I neither like nor dislike”. Firmness and odor had higher values of acceptance than color. For 25 and 12.5% of the panelists, firmness and odor were rated as “I like it a lot”, respectively, and 56.3% of the panelists conferred the label of “I like it” to both attributes. Cheese color was the lowest rated attribute, given that 12.5% of the panelists chose “I like it a lot” and 25% for “I like it”.

RESUMEN

Palabras clave:
Queso
Pericarpio de cacao
Cacao fino y de aroma
Curva de crecimiento
Compuestos fenólicos
Análisis sensorial

El pericarpio del cacao es un subproducto generado en la transformación agroindustrial, comúnmente subutilizado, a pesar de los compuestos fenólicos presentes en él que pueden ser una alternativa para preservar la calidad microbiológica de los alimentos. El objetivo del presente trabajo fue evaluar el poder inhibitorio *in vitro* de compuestos fenólicos provenientes del pericarpio de cacao frente a *Salmonela spp.*, comúnmente presente en queso fresco producido en Manabí, Ecuador; así como el efecto en las características sensoriales del queso después de su inmersión en una solución de compuestos fenólicos. Los análisis microbiológicos *in vitro* del poder inhibitorio de los compuestos fenólicos mostraron que concentraciones de 1 and 1,5% tuvieron las mayores zonas de inhibición de *Salmonela* spp., con medias de 10,67 mm y 11,8 mm de diámetro, respectivamente. Por otro lado, la curva de crecimiento de *Salmonela* spp. indicó que se requirió de 2 h para una inhibición completa de la bacteria frente a concentraciones de compuestos fenólicos de 1 y 1,5%. Los análisis sensoriales del queso tratado con compuestos fenólicos presentaron que la firmeza y el olor tuvieron mejores calificaciones que el color. La firmeza y el olor se calificaron con “me gusta mucho” por 25% y 12,5% de los panelistas, respectivamente y con “me gusta” por un 56,3% de los panelistas para ambos atributos. El color del queso fue el atributo de menor calificación, con 12,5% de los panelistas que escogieron el nivel de agrado “me gusta mucho” y 25% para el nivel “me gusta”.

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Cocoa tree is cultivated in tropical countries with hot and humid environments. Three countries, Cote D'Ivoire, Ghana and Indonesia together cultivate 61% and produce 67% of global traded cocoa (Campos-Vega *et al.*, 2018), considering the top ten countries account for approximately 93% of total world cocoa production (Table 1). The average yield in countries from Latin America and the Caribbean is 0.39 t ha^{-1} of cocoa and in the world is 0.44 t ha^{-1} of cocoa (Sánchez *et al.*, 2019). The main varieties of cocoa produced and sold in Ecuador are cacao fino y de aroma (fine aroma cocoa) (Clones Nacionales, Pichilingue Tropical Experimental Station, Ecuador) and CCN-51 cocoa (Castro Naranjal Collection, Guerrero, 2015). Ecuador is an important producer of fine aroma cocoa worldwide and is currently recognized for providing more than 60% of its production. Fine aroma cocoa is recognized worldwide for its aroma, color and quality. The use of the fruit seed to produce chocolate, cosmetics, among others, triggers the generation of by-products. The agroindustry of cocoa generates between 17 and 20 kg of by-products for each 100 kg of cocoa fruit (López, 2014), which currently are used in soil fertilization (Munongo *et al.*, 2017). Nonetheless, several phenolic compounds were identified by LC-MS/MS in cocoa pod-husk by Karim *et al.* (2014) such as phenolic acids, flavonoids, luteolin, apigenin and linarin. Despite cocoa pod-husk is an important source of phenolic compounds (Hii *et al.*, 2009), with great potential for application in the field of nutrition, health and medicine, due to its antioxidant and antimicrobial characteristics (Niemenak *et al.*, 2006), in many cases the by-products of cocoa are accumulated without any treatment, leading to the generation and transmission of diseases that may affect crops (FEDECACAO, 2013).

Table 1. Countries with the highest production of cocoa (Campos-Vega *et al.*, 2018).

	Production (t)	% total	Cultivation (ha)	% total
Cote D'Ivoire	1,472,313	32.96	2,851,084	27.96
Ghana	858,720	19.23	1,683,765	16.51
Indonesia	656,817	14.71	1,701,351	16.69
Cameroon	291,512	6.53	723,853	7.10
Nigeria	236,521	5.30	838,046	8.22
Brazil	213,843	4.79	720,053	7.06
Ecuador	177,551	3.98	454,257	4.45
Peru	107,922	2.42	125,580	1.23
Dominican	81,246	1.82	172,940	1.70
Colombia	56,163	1.26	165,844	1.63

The fresh cheese is an Ecuadorian artisanal cheese with 37% moisture (Espinosa, 2012) and good acceptance among consumers, especially in the province of Manabí (Ecuadorian coast). The presence of pathogenic microorganisms such as *Salmonella* in this kind of cheese (Zambrano, 2014) together with commercialization of the cheese at inappropriate temperatures can affect the health of the consumer (Lee *et al.*, 2015) and contribute to a high number of people suffering from Salmonellosis in the province of Manabí, Ecuador (Ministerio de Salud Pública, 2014, 2015, 2016, 2017, 2018).

Salmonella and other pathogenic microorganisms have been found in cheese of countries such as Colombia (Instituto Nacional de Salud, 2011), Turkey (Kahraman *et al.*, 2010), France (Domínguez *et al.*, 2009), Germany (Fretz *et al.*, 2010) and Egypt (El-Baz *et al.*, 2017). To deal pathogen microorganisms, natural antimicrobials can be an important alternative to preserve the microbiological quality of food and guarantee consumer safety (Abdalla *et al.*, 2007).

Concerns about the use of synthetic chemical antimicrobials have renewed the interests of consumers using natural and safe alternatives (Nazzaro *et al*., 2009). The antimicrobial activity of phenolic compounds is well documented by several authors (Erdemoglu *et al*., 2007; Xia *et al*., 2011). The growth of strains of *Salmonella* and *Escherichia coli* is inhibited by phenolic compounds extracted from vegetables, fruits, herbs and spices (Cetin-Karaca and Newman, 2015), cocoa bean (Todorovic *et al*., 2017) and bean shells (Nsor-Atindana et al., 2012). However, no studies of the use of phenolic compounds of cocoa pod-husk against *Salmonella* growth have been performed.

In the present work, the inhibition of *Salmonella* spp. in fresh cheese was studied, after immersing the cheese in a solution of phenolic compounds previously extracted from the pericarp of fine aroma cocoa. Additionally, sensory analyzes were used to examine whether cheese treated with phenolic compounds showed organoleptic differences compared to an untreated cheese.

MATERIALS AND METHODS

Samples of fine aroma cocoa pericarp (*Theobroma cacao* L.) were collected at the Alegría farm, located in Junín, Manabí province, Ecuador, with 0.9277 south latitude and 80.2058 western longitude. The samples were washed with water and dried in an oven at 50 °C, ground and stored for future analyzes. Fresh cheese was purchased at the Nuevo Tarqui shopping center in the city of Manta, Ecuador.

Alcoholic extraction of phenolic compounds

The extraction of phenolic compounds was done according to Santacruz *et al.* (2020). An amount of 15 g of the previously ground cocoa pericarp was mixed with 150 mL of ethanol (95% v/v) and kept under stirring at 130 rpm for 24 h at 25 °C. Afterwards, the sample was centrifuged (SIGMA 2-16P, Germany) for 10 min at 4000 rpm and the supernatant was filtered by vacuum, saving the filtered liquid fraction.

Quantification of phenolic compounds

The quantification of phenolic compounds was done according to the Folin-Ciocalteu method proposed by Mahmood *et al*. (2011). A stock solution was prepared by mixing 10 mL of the previous filtered fraction with 5 mL of ethanol (95% v/v) and distilled water to complete a total volume of 100 mL. From the stock solution, 0.1 mL was mixed with 0.5 mL of the Folin-Ciocalteau reagent, allowing it to stand for 5 min. Afterwards, 1 mL of a sodium carbonate solution (5%) was added and made up to 25 mL with distilled water. The resulting solution was left in dark for 1 h and its absorbance at 760 nm was measured (Spectrophotometer Jenway 6320D, China). The quantification of phenolic compounds was performed using a calibration curve with gallic acid standards, and the results were expressed in mg of gallic acid equivalent (GAE) g^{-1} cocoa pericarp.

Inhibition of *Salmonella* spp. growing by phenolic compounds

The antibacterial activity of phenolic compounds against *Salmonella* spp. was performed according to Santacruz and Castro (2018) and the Clinical and Laboratory Standards Institute (CLSI, 2009). *Salmonella* spp. was inoculated into Petri dishes using Salmonella-Shigella agar (HiMedia Laboratories, India) as culture medium and incubated at 37 °C for 24 h. Filter paper disks (Fisher Scientific Q2) of 5 mm diameter were immersed in a solution of phenolic compounds at a specific concentration (see experimental design). The disks were dried and placed in the center of the Petri dishes containing *Salmonella* spp. and incubated at 37 °C for 24 h. Afterwards, the zone of inhibition of the growth by phenolic compounds was measured in triplicate.

Bacterial growth curve measurement

Bacterial growth was performed according to Puupponen *et al.* (2001). 10 mL of fresh growth medium (Salmonella-Shigella agar) were inoculated with 5% of culture (frozen *Salmonella* spp. was transferred to liquid media and incubated for 24 h). Phenolic compounds, from alcoholic extraction, were added to the culture media to give final concentrations of 0.5, 1 or 5%. The culture was manually shaken, and afterwards, it was incubated at 37 °C for 24 h. Bacterial growth was followed by taking seven samples from the culture during the incubation period of 24 h (0, 0.5, 1, 2, 4, 6, and 24 h). The samples were plated in Petri dishes containing Salmonella-Shigella agar and incubated at 37 °C for 24 h before bacterial count was recorded.

Bacterial growth in cheese

Fresh cheese was immersed in a 1% phenolic compounds solution for 15 min. Afterwards, cheese samples were stored for 8 days, overnight under refrigeration conditions and during the daytime at room temperature (approximately 25 °C).

Microbiological analysis of *Salmonella* in cheese was performed on samples after 0, 2, 4, 6 and 8 days of storage, according to ISO 6579: 2002 method (ISO, 2002). For the test, 10 g of cheese were weighed and placed in a sterile resealable plastic bag, before adding 90 mL of sterile peptone water. The sample was homogenized manually, and 1 mL of the liquid was transferred to a glass test tube before adding 9 mL of peptone water (10^{-1} dilution). The process was repeated to get a dilution of 10^{-4}, and immediately 1 mL of the solution was inoculated into Petri dishes and incubated at 37 °C for 24 h prior CFU counting.

Sensory analyzes

Cheese samples were previously analyzed to verify the absence of *Salmonella*. Afterwards, the cheese was immersed for 15 min in a 1% phenolic compounds solution before sensory analyzes. First, a duo-trio test ISO 10399: 2004 (ISO, 2004) with 16 semi-trained panelists was done to determine organoleptic differences between cheese samples treated with phenolic compounds and a control sample (cheese with no immersion). Subsequently, attributes of firmness, color, odor and flavor were evaluated using a five-point hedonic scale, with scores ranging from “I like it a lot” (5) to “I dislike it a lot” (1).

Texture analysis

Puncture tests were performed according to Castro *et al.* (2017), using cheese cubes of edge 2 cm, with slight modifications. A Shimadzu texturometer (Model EZ-LX, Japan) together with a stainless-steel probe of 3 mm diameter and 8 cm length were utilized. The probe speed was 10 mm s^{-1} and the maximum penetration force was recorded. Results were the average of three measurements.

Experimental design and statistical analyzes

A completely randomized design with a unifactorial arrangement was used. The concentration of phenolic compounds obtained by alcoholic extraction at 3 levels (0.5, 1 and 1.5%) was established as the independent variable (Medrano, 2019), whereas the zone of inhibition and the bacterial growth measurement were the dependent variables. The concentration of phenolic compounds with the best *Salmonella* inhibition was applied to cheese samples, before sensory analysis.

ANOVA and the significance of the difference between means was determined by Tukey test (P<0.05) using InfoStat statistics software (Infostat version 2014, Argentina). All measurements were performed in triplicate.

RESULTS AND DISCUSSION

The results of the inhibition of the growth of *Salmonella* spp. by phenolic compounds showed that there was a difference between *Salmonella* inhibition zones at the three concentrations of phenolic compounds (P<0.05) with values between 7.78 and 11.78 mm (Table 2). A positive correlation was also found between the inhibitory effect and the concentration of phenolic compounds by Hernández *et al.* (2012). The results of the present study agree with those obtained by Nsor-Atindana *et al.* (2012) for an ethanolic extract of cocoa pericarp at 100 mg mL^{-1} with zones of inhibition between 10.98 and 12.21 mm against *Salmonella*, *E. coli, S. aureus* and *B. cereus*. The inhibitory effect can be influenced by the type of phenolic compounds (Orihuela, 2016) that acts to a greater or lesser degree on the cell wall of bacteria, resulting in the loss of cell structure and the death of the microorganism. Another explanation for the inhibitory effect may be the diffusion

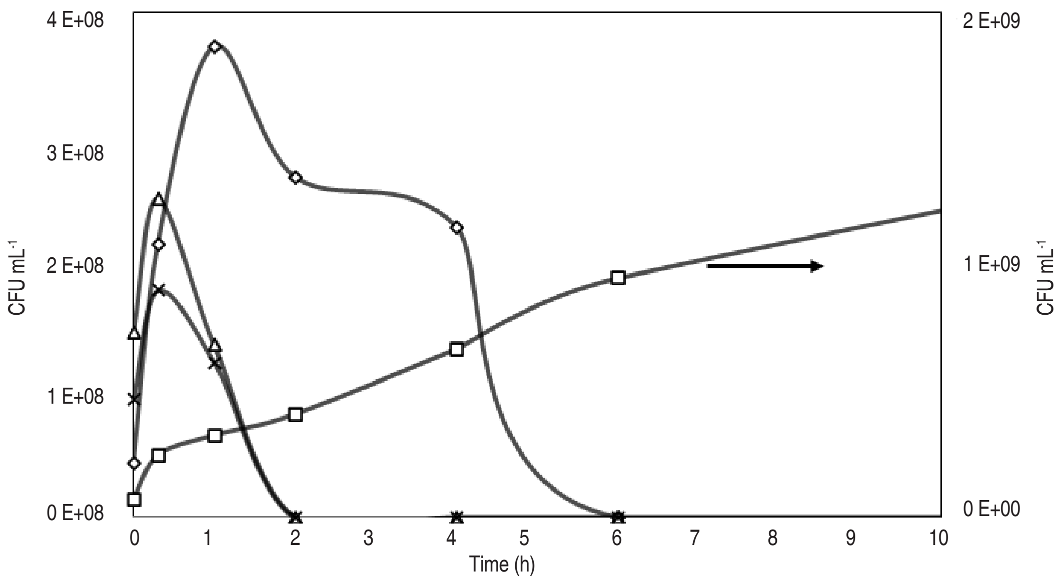
Table 2. Inhibition of growth of *Salmonella* spp. by phenolic compounds extracted from the pericarp of cocoa.

Phenolic compounds in solution (%)	Diameter of inhibition zone (mm)
0.5	7.78±1.15 a
1.0	10.67±1.32 ab
1.5	11.78±1.86 b

Mean value and standard deviations of measurements made with nine replicates. Different letters denote significant differences (P<0.05).

of hydrophilic compounds through the channel proteins of the outer membrane that surrounds the cell wall of gram-negative bacteria (Santos *et al*., 2014) This cell wall may contribute to the entry of phenolic compounds, which may have resulted in the observed cell death.

The measurement of bacterial growth curve (Figure 1) shows an increase in CFU mL^{-1} for the control sample along storage time. A complete inhibition of *Salmonella* spp. was observed after 2 h in the presence of phenolic compounds at 1 and 1.5%. The concentration of 0.5% showed a complete inhibition of *Salmonella* after 6 h. Phenolic compounds presented a dose-dependent antibacterial activity (Khan *et al*., 2021). Puupponen *et al*. (2001) showed that berry extracts at concentrations between 1 and 5 mg mL^{-1} were strong inhibitors of *Salm. enteric*. The inhibitory effect over time may be due to affectations of the bacteria cell wall because of the presence of the phenolic group (Puupponen *et al*., 2001). However, diffusion of phenolic compounds through the cell wall may allow an initial growth of bacteria (Figure 1) (Khan *et al*., 2021).



□ 0%, ◊ 0.5%, Δ 1%, × 1.5%

Figure 1. Curve of *Salmonella* growth in the presence of solutions of phenolic compounds.

The control sample (cheese with no immersion) showed presence of *Salmonella* spp. According to Ecuadorian regulations, NTE INEN 1529-15: 2013 (INEN, 2013), *Salmonella* should not be present in cheese. Cheese samples previously immersed in phenolic compound solutions at 1% showed an absence of *Salmonella* at 0 day of storage. In fact, Figure 1 shows no presence of *Salmonella* in cheese treated with phenolic compounds at 0.5% after 2 h of storage.

Sensory analyzes

Results of the duo-trio test showed that 15 of 16 panelists did not find difference between cheese previously immersed in a solution of phenolic compounds and the control sample. Texture analysis displayed that penetration force of control sample was 1.02 N, which was lower than cheese treated with phenolic compounds, showing values between 1.92 and 1.99 N (P<0.05). Instrumental texture of cheese does not have a single dominant characteristic and exhibit at least two primary characteristics (e.g. chewiness and cohesiveness) (Meullenet *et al*., 1998).

Figure 2 shows the responses of the panelists to the five-point hedonic test about cheese treated with phenolic compounds. Firmness and odor obtained the highest ratings among the evaluated characteristics, since 56.3% of the panelists classified them as "I like it". For the flavor, 37.5% of the panelists gave a rating of "I like it" and an equal percentage for the color of the cheese with the label of "I neither like nor dislike". The lower color rating could be due to the fact that phenolic compounds developed a yellow color in cheese, color that did not change along storage.

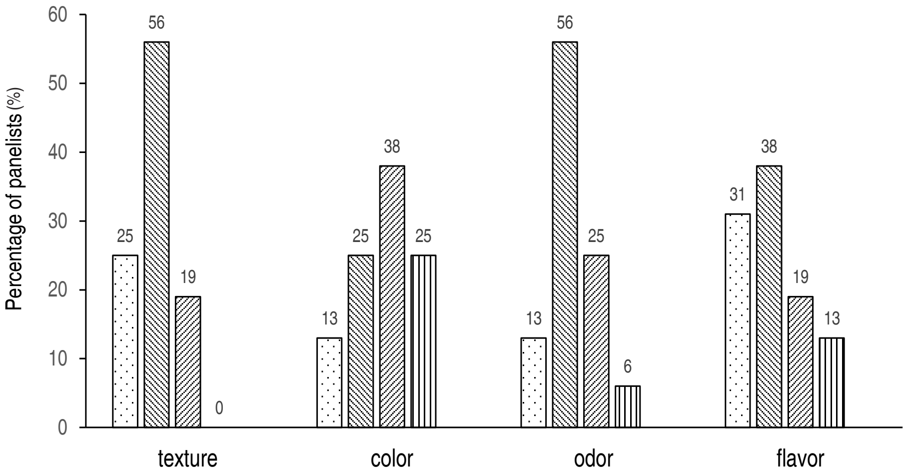


Figure 2. Sensory analyzes. Percentage of panelists and their evaluation of firmness, color, odor and flavor on cheese using a five-point hedonic scale, with scores ranging from "I like it a lot" , "I like it", "I neither like nor dislike", "I dislike it" ,"I dislike it a lot" .

Based on present results, it is difficult to conclude if color had influence either in odor or flavor. Moreover, visual and auditory appreciations can modify the flavor of food but are not intrinsic to it (Auvray and Spence, 2008). Koch and Koch (2008) stated that "In fact, it may be that color has nothing to do with the taste of food or drink". Meanwhile, Bayarri *et al.* (2001) suggested that "the possible influence of color on flavor perception is under discussion and no clear conclusions have been attained yet".

CONCLUSIONS

The solutions of phenolic compounds at concentrations of 1 and 1.5% showed the highest *in vitro* inhibition of *Salmonella* spp., requiring 2 h to achieve a complete inhibition of the bacteria.

Sensory analysis showed there was no difference between the cheese treated with phenolic compounds and a control cheese. Firmness and odor had high ratings whereas color had the lowest rating among the evaluated attributes.

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