PRESENCE OF SALMONELLA SPP. IN FOOD FROM THE ECUADORIAN COAST AND ITS INHIBITION BY LACTIC ACID BACTERIA OR PHENOLIC COMPOUNDS FROM CACAO POD HUSKS

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Abstract - Phenolic compounds have been identified in several agricultural by-products, i.e. cacao pod husks, that are normally wasted or subtilized. Phenolic compounds and lactic acid bacteria (LAB) showed inhibition properties against bacteria. However, their utilization in food may be reduced since LAB and phenolic compounds may be affected by environmental conditions. Regions in Ecuador, specially Manabí province, reports high number of Salmonellosis cases. The present work examines the presence of Salmonella spp. in artisanal and street food in the city centre of Manta (Manabí province, Ecuador). Additionally, encapsulated L. acidophilusor phenolic compounds from cacao pod husks applied toartisanal cheeseas control of Salmonella spp. was studied. Results showed that L. acidophilus and phenolic compounds from cacao pod husks showed microbial activity against Salmonella.

Key words - Salmonella, cacao pod husks, phenolic compounds, lactic acid bacteria

I. INTRODUCTION

Cacao by-product is a solid waste product of chocolate processing. Cocoa pod husks contain 7.7 crude protein, 4.4 ether extract, 32.5 crude fibre, 10.1 ash and 10.8% hemicellulose (%, w/w d. b.) (Donkoh& Adomako, 1991). Thousands of tonnes of cacao by-product are produced each year, these waste products are often dumped in landfill.Extraction of the phenolic compounds from cacao by-products has the potential to somewhat limit the environmental damage that can be caused by this waste fraction and may even provide an additional source of income for cacao producers.

Phenolic compounds are one of the secondary metabolites that naturally occur in small quantities in plants. Phenolic compounds comprise flavonoids, phenolic acids, and tannins, among others.In the last few years, great attention has been paid to phenolic compounds due to their ability to promote benefits for human health, such as the reduction in the incidence of some degenerative diseases like cancer (Conforti et al., 2009), antioxidant and anti-inflammatory effects (Balasundram et al. 2006; Ham et al. 2009), among others.

The processing of plant foods results in the production of by-products that are rich sources of bioactive compounds, including phenolic compounds (Schieber et al., 2001). Phenolic compounds with antioxidant activity have been identified in several agricultural by-products, such as rice hulls (Ramarathnam et al., 1989), buckwheat hulls (Watanabe et al., 1997) and oat hulls (Bryngelsson et al., 2002). Phenolic compounds and lactic acid bacteria (LAB) showed inhibition properties against bacteria (Borges et al., 2013;Rokka and Rantamaki, 2010). However, LAB show low survival rates in food products (Husmaini et al., 2011; Ashraf and Smith, 2016), and therefore encapsulation may help to preserve their viability (Mirzaei et al., 2012).

Ecuadorian Ministry of Health has a register of diseases from food intake among others. A high number of people suffering from Salmonellosis has been identified in Ecuador year after year. Only on the last three years, between 1710 and 3373 cases were identified (Ministerio de SaludPública, 2016).Within Ecuador, Manabí province showed the highest percentage of Salmonellosis cases. Processing and commercialization conditions of artisanal and street food may not fulfill Ecuadorian regulations, leading to the presence of pathogen microorganisms like Salmonella spp. in artisanal cheese (Zambrano, 2014).
Control of pathogen microorganisms in cheese has been studied previously using LAB (Coelho et al., 2014; Mirzaei et al., 2012). L. acidophilus is the major bacteria present in the human gastrointestinal tract(Webb, 2007), therefore it may be interesting to know the control of Salmonella spp. using L. acidophilus.
Based on the previous information, the present work examines the presence of Salmonella spp. in food commercialized in the city centre of Manta (Manabí, Ecuador). Additionally,L. acidophilusor phenolic compounds from cacao pod husks were used as control agent of Salmonella spp. in artisanal cheese stored at 4 °C.

II. MATERIALS AND METHODS

L. acidophilus frozen cultures were obtained from Chr. Hansen A/S (Denmark). LAB was defrosted and activated in glucose solution (0.02 g/ml) for 24 h.

Sodium alginate from Shandong Jeijing Group Corporation (China).

Cacao pod husks were obtained from cacao fruits (cv. CCN-51, Instituto Nacional de Investigaciones Agropecuarias, Ecuador) in Calceta, Ecuador. Soluble solids from cacao pod husks were measured and reported as º Brix.

Food samples for identification of presence of Salmonella were chosen previously.Samples oflonganiza, marinated fish (ceviche), artisanal cheese, mortadella, grilled chicken, meringue for bakery and commercial ice were purchased from restaurants or stores from the city centre of Manta, Ecuador. Potable water was collected from houses located in the city centre of Manta.

2.1. Presence of Salmonella in foods

The analyses to determine presence of Salmonella were done according to the norm NTE INEN (1996). 25 g of sample were inoculated with 225 ml peptone solution and incubated at 37 C for 2 h. 1 ml of the prepared sample was inoculated in Rappaport and tetrathionate solution. Afterwards, the solutions were plated in Salmonella-Shigella agar, incubated at 37 C for 24 h and Salmonella colonies were identified visually.

2.2. Extractionofphenoliccompounds

The extraction of phenolic compounds was carried according to the method proposed by Slinkard and Singleton (1977). Cacao pod husks were dried in an oven (Precision, USA) at 50 °C. After drying, samples were milled (Corona mill, Colombia) and particles above 1 mm were discarded. The powder sample (15 g) was dissolved in 150 mL of ethanol (95% v/v) and stirred for 24 h at 20ºC.Afterwards, the mixture was filtered, and the filtrate was concentrated in a rotary evaporator (Buchi, Switzerland) until a dry residue was obtained.

2.3. Encapsulation of Lactobacillus acidophilus and phenolic compounds

Encapsulation was performed in an experimental device, composed of two cylindrical vessels connected with a valve. The method is a combination of ionic gelation and emulsification (Vemmer and Patel, 2013). Capsule formation was performed on the upper vessel by adding drop by drop 6 mL of 1.8 % w/v Na-alginate solution containing either 7 x 10^7 CFU/mL of L. acidophilusor an alcoholic extract containing phenolic compounds, to a mixture of palm oil (La Fabril, Ecuador) and $CaCl_2$ solution (100:2 volume ratio of palm oil/0.1 M $CaCl_2$) which was kept on stirring at 500 r.p.m. (propeller stirrer, Fisher Scientific BDC2002, Canada). Afterwards, the valve located between the two vessels was opened, allowing the formed capsules to move to a lower vessel containing $CaCl_2$ solution. After capsules sedimentation, they were collected for further analyses.

2.4 Application of Lactobacillus acidophilus and phenolic compounds to artisanal cheese

Either L. acidophilus or phenolic compounds were added to the surface of cheese samples within an edible film. Film preparation was performed according to Santacruz et al. (2015). Cassava starch suspension (1% w/w) was stirred and heated from 25 °C to 90 °C. Afterwards, the solution was cooled to 30 °C, homogenized with an ultraturrax (Polytron, Switzerland) and L. acidophilus(either free or encapsulated) or phenolic compounds (encapsulated) were added. Twenty mL of a 1% (w/v) starch solution, containing 7.2 x 10^7 CFU/mL of L. acidophilus or 6mL of encapsulated phenolic compounds, was transferred to a 9.5 cm diameter Petri dish and incubated for 30 days at 25 °C and 60% relative humidity. Determination of viable cells in the film was performed along 30 days of storage. A film fragment was used to prepare a bacterial suspension with an absorbance between 0.08 and 0.1 measured at a wavelength of 625 nm (Medina et al., 2005). The suspension corresponded to an equivalent of $1x10^8$ CFU/mL. Released L. acidophilus was 10 times serially diluted with KCl solution and 100 µL aliquots were plated on MRS agar plate. Colonies of L. acidophilus were determined according to Shi et al. (2013) after aerobic incubation at 37 °C for 48 h.

Cheese samples were dipped into the starch film solutions containing L. acidophilus or phenolic compounds. Afterwards, coated cheese was allowed to dry at room temperature and transferred to plastic bags and stored for 30 days at 4 °C. Determination of mesophilic aerobic bacteria on cheese was done after 10, 20 and 30 days of storage (Castro et al., 2014).

2.5 Inhibition of Salmonella spp. growing by Lactobacillus acidophilus or phenolic compounds

Anti-bacterial activity of eitherL. acidophilusor phenolic compounds was determined according to CLSI (2009) and was carried out on Salmonella strain previously isolated from artisanal cheese. Salmonella spp. was inoculated in Petri dishes using Salmonella-Shigella agar culture medium (HiMedia Laboratories, India) and incubated at 37 °C for 2 days. Filter paper disks (Fisher Scientific Q2) of 5 mm diameter were dipped into a solution containing L. acidophilus($3x10^8$ CFU/ml) or alcoholic extract. The disks were placed in the center of a Petri dish containing Salmonella spp. and incubated at 37 °C for 2 days. The inhibition zone was measured after 24 and 48 hours in triplicate.

Migration of LAB from capsules to the surroundings was analyzed by taking a sample from the zone of inhibition and using as inoculum in MRS agar plate. Microbial growth was verified with incubation at 37 °C for 2 days.

Presence of bacteriocins was analyzed by incubating L. acidophilusin glucose solution (0.02 g/ml) for 24 h. Afterwards the sample was centrifuged at 1792 x g

for 10 min (centrifuge, Sigma, Germany) and the supernatant was neutralized with NaOH (Rogers and Montville, 1991). The neutralized solution (BLA) was analyzed as anti-bacterial agent against Salmonella by measuring an inhibition zone, as previously described.

2.6 Statistical analysis

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

III. RESULTS AND DISCUSSION

Table 1 show that four of the eight food samples contained Salmonella. Potable water had the highest presence of Salmonella with 14.3%, followed by artisanal cheese (7.1%), marinated fish (3.6%) and longaniza (2.8%).

There are very few studies in Ecuador about the presence of Salmonella in food. This information may help to public health to prevent and control salmonellosis in Ecuador by improving processing and storage conditions of commercialized food.
The results of the present work are similar to previous studies. Yánez et al. (2008) found 5.3% cheese samples contaminated with Salmonella whereas7.9% of cheese samples were positive in a study of Durango et al. (2004). However, sausages from a different kind (chorizo) showed higher values, 7.9% (Durango et al., 2004) and 12.35 (Yánez et al., 2008).

Table 1. Presence of Salmonella in food commercialized in the city centre of Manta

Food	Numberofsamples	Presence of Salmonella (%)
Longaniza	36	2.8
Marinatedfish	28	3.6
Ecuadorianartisanalch	28	7.1
Potable water (Manta,	21	14.3
Commercial ice	9	0
Mortadella	36	0
Grilledchicken	27	0
Meringueforbakery	27	0

Table 2 shows that encapsulated L. acidophilus had a higher viability than free cells (FLAB, cells within a film) along storage ($p < 0.05$). Viability of both free and encapsulated cells decreased along storage, being zero for free L. acidophilus after 20 days of storage. Cell encapsulation may show a protective effect compared to uncoated cells (Sathyabama et al., 2014; Kiran et al. 2015). A different media where encapsulated LAB was suspended may led to higher viable counts compared to free cells. Additionally, a low water activity of the starch film may promote growing of bacteria at a lower rate in FLAB (Fennema, 1985).

Table 2. Viability of free and encapsulated L. acidophilus incorporated to cassava starch films and stored at 25 ºC

Sample	Day 0	Day 10	Day 20	Day 30
	Log CFU/g	Log CFU/g	Log CFU/g	Log CFU/g
FLAB[1]	12.03 ± 0.10^{A}	5.42 ± 0.04^{A}	0.00^{A}	0.00^{A}
ELAB[2]	12.16 ± 0.16^{A}	10.35 ± 0.05^{B}	9.79 ± 0.02^{B}	5.65 ± 0.03^{B}

n = 3

[1] FLAB = cassava starch film containing free L. acidophilus

[2] ELAB = cassava starch film containing encapsulated L. acidophilus

Values with different superscript letters show statistical difference ($p < 0.05$)

Uncoated cheese showed higher viable counts (mesophilic aerobic bacteria) compared to both coated samples ($p<0.05$, Table 3). A decreased of viable counts along the whole storage was only observed for FELAB. No presence of LAB from day 20 to day 30 (FLAB, Table 2) may lead to an increase of viable counts for FFLAB from 4.1 CFU/g to 6.1 CFU/g (Table 3). The presence of LAB may help to inhibit microorganisms by producing bacteriocins (Aymerich et al., 1996). This fact is supported by results from activity of encapsulated L. acidophilus against Salmonella spp. where no migration of LAB from capsules to the surroundings was found, however a zone of inhibition was observed. Additionally, a zone of inhibition of 1.1 cm diameter was formed after 24 h of adding a BLA solution. Therefore, the inhibition may be the result of bacteriocins and not only by acidification.In vitro studies showed inhibition of Salmonella spp. growing by L. acidophilus.Zones of inhibition of 2.08 and 2.11 cm diameter were formed after 24 and 48 h of adding L. acidophilus respectively. Previous studies showed the inhibition of E. coli, L. monocytogenes and S. enteritidis by LAB (Winkowski et al., 1993 and Lord, 2002).

Table 3. Microbiological results (mesophilic aerobic bacteria) of Manaba cheese coated with cassava starch films containing either free or encapsulated L. acidophilus stored at 4 ºC.

Sample	Day 0	Day 10	Day 20	Day 30
	Log CFU/g	Log CFU/g	Log CFU/g	Log CFU/g
FFLAB[1]	8.60 ± 0.08^{A}	4.10 ± 0.15^{A}	6.10 ± 0.13^{A}	6.40 ± 0.15^{B}
FELA	8.60±0.	5.90±0.	5.90±0.1	3.20±0.1

B[2]	08[A]	15[B]	3[A]	5[A]
Uncoated	8.60±0.08[A]	9.20±0.15[C]	10.23±0.13[B]	10.80±0.15[C]

n = 3

[1] FFLAB = Artisanal cheese coated with cassava starch film containing free L. acidophilus

[2] FELAB = Artisanal cheese coated with cassava starch film containing encapsulated L. acidophilus

Values with different superscript letters show statistical difference ($p < 0.05$)

Coated cheese with encapsulated phenolic compounds showed a reduction of viable counts (Salmonella spp.) from 5.8 CFU/g to 4.4 CFU/g (day 0 to day 5). However, an increase of viable counts was observed at day 10.The microbial activity of phenolic compounds against Salmonella was shown by inhibition analyses where a zone of inhibition of 1.4 cm diameter was formed after 24 h of adding encapsulated phenolic compounds. Antimicrobial activities of phenolics products can involve various modes of action, i.e. enzyme inhibition by the oxidized products, possibly through reaction with sulfhydyl groups or through more nonspecific interactions with the proteins(Borges et al., 2013).Since antimicrobial activity may be the result of chemical reactions where phenolic compounds participate, adecrease of concentration or even more, absence of phenolic compounds may lead to an increase of viable counts.

CONCLUSION

Foods that are not properly processed or stored could be responsible of Salmonella contamination in Manta, Ecuador. Encapsulated L. acidophilusshowed higher viability than free cells during storage at 25 °C. L. acidophilus and phenolic compounds from cacao pod husks showed microbial activity against Salmonella. Edible films containing encapsulated L. acidophilus may be a promising tool to control mesophilic aerobic bacteria during storage of Ecuadorian artisanal cheese at refrigeration conditions.

REFERENCES

[1] Anabela Borges, Carla Ferreira, Maria J. Saavedra, and Manuel Simões. “Antibacterial Activity and Mode of Action of Ferulic and Gallic Acids Against Pathogenic Bacteria”. Microbial Drug Resistance 19, 256, 2013

[2] Ashraf, R. & Smith, S. “Commercial lactic acid bacteria and probiotic strains- tolerance to bile, pepsin and antibiotics”. International Food Research Journal, 23, 777-789, 2016

[3] Asunción R. “Atención farmacéutica en la enfermedad periodontal”. Plantas medicinales 1, 24-36, 2010

[4] Aymerich, T., Holo, H., Håvarstein, L.S., Hugas, M., Garriga, M., Nes, I.F. “Biochemical and genetic characterization of enterocin A from Enterococcus faecium, a new antilisterial bacteriocin in the pediocin family of bacteriocins”. Appl. Environ. Microbiol. 62, 1676–1682, 1996

[5] Balasundram N, Sundram K, Samman S.“Phenolic compounds in plants and agroindustrial by-products: antioxidant activity, occurrence, and potential uses”. Food Chem 99:191–203, 2006

[6] Borges A, Ferreira C, Saavedra MJ, Simões M. “Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria”. Microb Drug Resist. 19, 256-65, 2013

[7] Bryngelsson, S., Mannerstedt-Fogelfors, B., Kamal-Eldin, A., Andersson, R., &Dimberg, L. H. “Lipids and antioxidants in groats and hulls of Swedish oats (Avena sativa L.)”. J. of the Science of Food and Agriculture 82, 606–614, 2002

[8] Castro, M., Rivadeneira, C., Mantuano, M.I., Santacruz, S., &Ziani, K. “Aplicación de recubrimientos comestibles a base de quitosano y Aloe vera sobre papaya (Carica papaya L. cv. “Maradol”) cortada”. Alimentos, Ciencia e Ingeniería, 22, 05-12, 2014

[9] Cisneros Domínguez G, Hernández Borges Y. “La educación para la salud bucal en edades tempranas de la vida”. Medisan 15, 1445-1458, 2011

[10] CLSI. “Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts”; Approved Guideline-Second Edition. CLSI document M44-A2. Wayne, PA: Clinical and Laboratory Standars Institute, 2009

[11] Coelho, M., Silva. C., Ribeiro. S., Dapkevicius. M.& Rosa, H. “Control of Listeria monocytogenes in fresh cheese using protective lactic acid bacteria”. Int. J. Food Microbiol.191, 53-9, 2014

[12] Conforti F, Menichini F, Formisano C, Rigano D, Senatore F, Arnold NA.“Comparative chemical composition, free radical-scavenging and cytotoxic properties of essential oils of six Stachys species from different regions of the Mediterranean Area”. Food Chem 116, 898–905, 2009

[13] Donkoh A., Adomako D. “Chemical composition of cocoa husk and its effect on growth and food efficiency in broiler chicks”. Animal Feed Science and Technology 35, 161-169, 1991

[14] Durango J., Arrieta G., Mattar S. “Presencia de Salmonella spp. en un área del Caribe colombiano: un riesgo para la salud pública”. Biomédica 24, 89-96, 2004

[15] Fennema, O. “Water and Ice”. In:Fennema. O. (Ed), Food Chemistry. Marcel Dekker: New York, 1985

[16] Ham S-S, Kim S-H, Moon S-Y, Chung MJ, Cui C-B, Han E-K. “Antimutagenic effects of subfractions of Chaga mushroom (Inonotus obliquus) extract”. Mutat Res Gen Toxicol, 672:55–9, 2009

[17] Husmaini, M., Abbas, E., Yuniza, A. &Alimon, A. “Growth and Survival of Lactic Acid Bacteria Isolated from Byproduct of Virgin Coconut Oil as Probiotic Candidate for Poultry”. International Journal of Poultry Science, 10, 309-314, 2011

[18] Kiran, F., Mokrani, M. &Osmanagaoglu, O. “Effect of encapsulation on viability of Pediococcuspentosaceus during its passage through gastrointestinal tract model”. Curr. Microbiol. 71, 95-105, 2015

[19] Lord, E. “Utilización de Lactobacillus acidophilus como control de Salmonella en pescado”. Thesis. Cartago: Instituto Tecnológico de Costa Rica, 2002

[20] Medina, A. Moreno, L. Velasco, M. & Gutiérrez, S. “Estudio comparativo de medios de cultivo para crecimiento y recuperación del Streptococcusmutans ATCC 25175 in vitro”. Nova 3, 1-120, 2005

[21] Ministerio de Salud Pública. “Subsistema de vigilancia epidemiológica SIVE–ALERTA”. Ministerio de Salud Pública, Ecuador, 2016

[22] Mirzaei, H., Pourjafar, H., &Homayouni, A. “Effect of calcium alginate and resistant starch microencapsulation on the survival rate of Lactobacillus acidophilus La5 and sensory properties in Iranian white brined cheese”. Food Chemistry, 132, 1966–1970, 2012

[23] NTE INEN 1529-15. “Control microbiológico de los alimentos”. Salmonella. Método de detección. Instituto Ecuatoriano de Normalización (INEN), 1996

[24] Ramarathnam, N., Osawa, T., Namiki, M., &Kawakishi, S. “Chemical studies on novel rice hull antioxidants. 2. Identification of isovitexin, a C-glycosyl flavonoid”. Journal of Agricultural and Food Chemistry 37, 316–319, 1989

[25] Rogers, A.M. and Montville, T.J. “Improved agar diffusion assay for nisin quantification”. Food Biotechnology 5, 161–168, 1991

[26] Rokka, S., &Rantamaki, P. "Protecting probiotic bacteria by microencapsulation: challenges for industrial applications". European Food Research and Technology, 231, 1–12, 2010
[27] Santacruz, S., Rivadeneira, C., & Castro, M. "Edible films based on starch and chitosan. Effect of starch source and concentration, plasticizer, surfactant's hydrophobic tail and mechanical treatment". Food Hydrocolloids, 49, 89-94, 2015
[28] Sathyabama, S., Ranjith, M., & Brindha, V. "Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in gastric environment". LWT - Food Science and Technology, 57, 419-425, 2014
[29] Schieber, A., Stintzing, F. C., & Carle, R. "By-products of plant food processing as a source of functional compounds – recent developments". Trends in Food Science and Technology 12, 401–413, 2001
[30] Shi, L., Li, Z., Li, D., Xu, M., Chen, H., Zhang, Z., & Tang, Z. "Encapsulation of probiotic Lactobacillus bulgaricus in alginate–milk microspheres and evaluation of the survival in simulated gastrointestinal conditions". Journal of Food Engineering 117, 99–104, 2013
[31] Slinkard K., Singleton V.L. "Total phenol analyses: automation and comparison with manual methods". American J. of Enology and Viniculture 28, 49-55, 1977
[32] Vemmer, M., & Patel, A. "Review of encapsulation methods suitable for microbial biological control agents". Biological Control 67, 380–389, 2013
[33] Watanabe M., Ohshita Y. &Tsushida T. "Antioxidant compounds from buckwheat hulls". J. of Agricultural and Food Chemistry 45, 1039-1044, 1997
[34] Webb, G. "Complementos nutricionales y alimentos funcionales". Spain:Acribia S.A., 2007
[35] Winkowski, K., Crandall, A., & Montville, T. "Inhibition of Listeria monocytogenes by Lactobacillus bavaricus in beef at refrigeration temperature". Applied and Environmental Microbiology, 59, 2552-2557, 1993
[36] Yánez E., Mattar S., Durango A. "Determinación de Salmonella spp. Por PCR en tiempo real y método convencional en canales bovinos y en alimentos de la vía pública de Montería". Asociación Colombiana de Infectología 12, 246-254, 2008
[37] Zambrano, M. "Determinación de la presencia de Salmonella en queso fresco comercializado en el cantón Chone provincia de Manabí entre Mayo y Julio del 2014". Thesis. Guayaquil: Universidad Católica de Santiago de Guayaquil, 2014

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