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Effect of agar-agar gelation on fermented coconut extract during *in vitro* digestion: probiotic survival and antioxidant activity

Efecto de la gelificación con agar-agar en un extracto de coco fermentado durante una digestión *in vitro*: supervivencia de probióticos y actividad antioxidante

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Technological innovation: Use of agar-agar as a gelling agent in a fermented non-dairy product.

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Resumen

El extracto de coco (CE) o comercialmente conocido como leche de coco puede actuar como medio de crecimiento para bacterias probióticas. Estos probióticos deberán sobrevivir durante su paso por el tracto digestivo y llegar a su sitio de acción. Por otra parte, el agar-agar (AA) es un agente gelificante que podría actuar brindando protección a biomoléculas, sin embargo, su efecto sobre la supervivencia de probióticos en alimentos aún no ha sido estudiado. El objetivo de este trabajo fue estudiar la habilidad del agar-agar para proteger bacterias probióticas y moléculas antioxidantes de un extracto de coco fermentado durante una digestión *in vitro*. Se llevó a cabo la fermentación utilizando un inóculo liofilizado (1% p/v) de *Bifidobacterium animalis ssp. lactis, Lactobacillus acidophilus y Lactobacillus delbrueckii* ssp. *bulgaricus* en CE hasta alcanzar un pH de 4.5. Se evaluaron dos concentraciones de AA (0.5 y 1.5% p/v) a fin de generar la gelificación del fermento. La digestión *in vitro* de los geles se llevó a cabo en la fase gástrica (GP) y fase intestinal (IP) mediante técnicas reportadas anteriormente. La supervivencia de los probióticos fue medida por la técnica de conteo en placa y la actividad antioxidante fue evaluada mediante los métodos de DPPH y ABTS. El contenido total de fenoles se llevó a cabo mediante el método de Folin-Ciocalteu. La mayor tasa de supervivencia de probióticos al final de la digestión simulada se observó en la

muestra con 0.5% AA ($1.7x10^8$±$8.8x10^7$UFC/g). Todas las muestras presentaron valores de supervivencia por arriba de los intervalos recomendados para un alimento probiótico (10^6 a 10^7 UFC/g). La muestra control (0% AA) presentó el mayor porcentaje de inhibición del radical ABTS (70.4±0.1%) al finalizar la IP, mientras que, los valores más bajos se observaron en la muestra con 1.5% AA (54.9±0.5%). Este comportamiento podría atribuirse a interacciones fisicoquímicas y/o efectos de obstrucción entre la matriz del AA y los compuestos antioxidantes. Un comportamiento similar se observó en el contenido de fenoles totales. Concentraciones superiores a 0.5 de AA podrían estar generando una sobreprotección de los probióticos, moléculas fenólicas y antioxidantes, y esto podría impactar positivamente en su supervivencia durante su tránsito por el sistema digestivo, permitiendo que alcancen su sitio de acción y/o absorción.

Palabras clave: Agar-agar, coco, digestión *in vitro*, probióticos.

Abstract

Coconut extract (CE) or commercially known coconut milk can be a growth medium for probiotic bacteria. These probiotics must survive during their passage through the digestive tract and reach their site of action. On the other hand, agar-agar (AA) is a gelling agent that could act by protecting biomolecules, however its effect on the survival of probiotics in food has not yet been studied. On the other hand, agar-agar (AA) is a gelling agent that may act as a biomolecule's protector but its effect on the survival of probiotics in food is not yet studied. The aim of this work was to study the ability of agar-agar to protect probiotics and antioxidant molecules in a fermented coconut extract during an *in vitro* digestion. Fermentation with a lyophilized inoculum mix (1% w/v) of *Bifidobacterium animalis* ssp. *lactis, Lactobacillus acidophilus y Lactobacillus delbrueckii* ssp. *bulgaricus* in CE was carried on until pH 4.5 was reached. Two concentrations of AA (0.5 and 1.5% w/v) were evaluated to generate the gelation of the ferment. *In vitro* digestion of the gels was performed considering the gastric (GP) and intestinal phase (IP) by techniques previously reported. Probiotic survival was measured by plate count and antioxidant capacity (DPPH and ABTS methods), as well as total phenols content (Folin-Ciocalteu method), were assayed. The highest survival of probiotics at the end of the simulated digestion was observed in the sample with 0.5% AA ($1.7x10^8$±$8.8x10^7$CFU/g). All samples presented survival values above the recommended intervals for a probiotic food (10^6 to 10^7 CFU/g). In concern to the antioxidant activity, the control sample (0% AA) presented the highest percentage of ABTS radical inhibition (70.4±0.1%) at the end of the IP, while the lowest values were found in the 1.5% AA sample (54.9±0.5%). This behavior could be attributed to physicochemical interactions or obstructive effects between the AA matrix and the antioxidant compounds. Similar behaviour was observed concerning total phenols. Concentrations of AA above 0.5% could be generating overprotection of probiotics, phenolic molecules, and antioxidants. This could positively impact their survival during their transit through the digestive system, allowing them to reach their site of action and/or absorption.

Keywords: Agar-agar, coconut, *in vitro* digestion, probiotics.

1. Introduction

Probiotics are live microorganisms which potentially confer a health benefit to the host when administered in adequate amounts: biomolecules production, improve intestinal microbiota, activate the immune system, reduce serum cholesterol and inhibit the growth of potential pathogens (1; 2; 3).

Fermented dairy products have been widely accepted as probiotic vehicles for consumers. 78% of world probiotic sales are destined for the yogurt industry (1). However, their survival is compromised due to the low pH of this type of food (4.2-4.6). Poor survival and low viability of probiotics in products such as yogurt is expressed in counts below 10^6 and 10^7 CFU/g. These values are recommended as the daily intake necessary to confer health (3).

Health benefits of probiotics were fulfilled by milk/other dairy products; however, lactose intolerance, cholesterol content and allergic milk proteins are limiting factors in the growth of dairy probiotics. Besides this with an increase in vegetarian consumers in both developed and developing countries, there is also a high demand for plant based probiotic products with good nutrients along with health promoting factors e.g. fruits, vegetables, cereal, and legumes (4).

Fermentation by different microorganisms is an effective technology that has been used not only to improve the flavor and stability of food but also to increase the bioactive and nutritional values original product.

Smaller molecules such as peptides, amino acids, and phenolic compounds, which possess physiological functions, are formed during fermentation. Antioxidant activity has been found in many fermented cereal, fruits and vegetable productions, proving that fermentation is an effective approach for the preparation of bioactive substances (5). For example, synthesis of vitamins (K and vitamins of group B), reduction of anti-nutrients in plant milk (tannins, phytates and cyanidins), generation of enzyme modulators such as acetylcholinesterase, glycosylated and amylases (6).

Due to this, the development of dairy-free yogurt-type products from soy milk represents a novelty in the field of fermented functional foods (1; 7; 8) or from coconut milk (2; 9; 10; 11; 12; 13; 14).

The aqueous extract of coconut or, commercially called coconut milk, is extracted of the solid coconut endosperm (*Cocos nucifera L.*) (10). Coconut has an estimated world production of 50–60 million tons per year (12). Mexico ranked in the 8° place in the world production. It was registered a production of 223,000 tons in 2017, being the state of Guerrero the first national producer (15). These statistical data make the coconut an ideal plant source with high expectations for its commercial exploitation in Mexico. The coconut extract is used as an ingredient for preparing traditional Asian dishes, in cakes, desserts, flavorings and even in cheeses. This significant participation in the food chain is because coconut milk confers creamy texture, smoothness, and interesting aromatic profiles to the food products. In addition, it does not contain lactose, which makes it an excellent alternative to dairy. Also, it is considered an easily digestible food with an abundant content of minerals (Ca, P and K), vitamins (B, C and E) and antioxidant compounds. Also, high content of lauric and oleic acid, which help prevent diseases such as atherosclerosis (2). Has been reported in the literature about the antihypertensive, antioxidative, antimicrobial, immunomodulatory, cardioprotective, and anti-inflammatory effects of coconut milk (16).

On the other hand, agar-agar is a non-toxic and biocompatible solidifying agent used as a culture medium for bacteria, fungi, and yeasts, as well as immobilization of enzymes and in packaging agents such as biofilm. It is the main structural polysaccharide of the cell walls of some species of red algae, such as *Gelidium* and *Gracilaria*. It is formed from repeating units of (i) agarose (gelling fraction), which is made up of repeating units of β-D-galactose and 3,6-anhydro-α-L-galactose and (ii) agaropectin (fraction not gelling) (11; 17). The most common use of agar in food is in the Japanese dessert Youkan, made from sugar and sweet red beans and agar in concentrations of 1.1%. Its use has also been reported in cakes, sauces, rotisserie chicken and bacon, mainly in Asia (18). It has been observed that by increasing the agar concentration, the gels show an increase in the fracture strength. This phenomenon also reduces the sensory perception of the sweetness of sucrose (19), which could also affect the release of other molecules present in the food (20).

Because of this, the aim of this work was to study the ability of agar-agar to protect the probiotics (*Bifidobacterium animalis* ssp. *Lactis*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), phenolic and antioxidant molecules in a fermented coconut extract during an *in vitro* digestion.

2. Material and methods

2.1. Biological material

The consortium of lyophilized microorganisms (SACCO) was used as inoculum, made up of *Bifidobacterium animalis* spp. *lactis*, *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* spp. *Bulgaricus*. The commercial coconut milk (Golden Star, Thailand) used as a fermentative medium was obtained from the local market. As the gelling agent was used the commercial agar-agar (Nature's blessings, Mexico) extracted from a red algae of the *Gelidium* species.

2.2. Methods

2.2.1. Formulation of the fermented coconut extract

The different treatments are presented in Table 1. First, a 75:25 coconut extract and water solution was prepared. A mixture of the inoculum [1% w/v] with the coconut extract was made and incubated in an oven set at 43°C until 4.5 pH was reached. After this, the agar-agar was added at the final concentration presented in Table 1 mixed with 100 mL of water. This solution was previously boiled for 2 min. Sample 3 without the addition of AA was prepared with the same procedure mixing the fermented CE only with water. The mixture was passed to containers and refrigerated until later analysis.

Table 1. Treatments used for *in vitro* digestion.

Sample	Agar-agar [% w/v]	Inoculum [% w/v]
1	1.5	1
2	0.5	1
3	0	1

2.2.2. Identification of bacterial growth in coconut extract by MALDI-TOF

A colony was isolated and placed on the MALDI plate with a sterile toothpick. It was then overlaid with 1 µL of saturated α-cyano-4-hydroxycinnamic acid (HCCA) as a matrix solution and allowed to dry. Each isolate was tested in triplicate. The loaded plate was placed on the instrument according to the manufacturer's instructions. Mass spectra were acquired automatically, within 5 to 10 minutes, in the positive linear mode at a laser frequency of 60 Hz with an acquisition range

of 1,960 to 20,000 Da. Spectra were imported into the integrated MALDI Biotyper software (version 3.1) and analyzed using standard pattern matching with a predetermined database.

2.2.3. Preparation of the extract

A 5 g aliquot of sample was macerated in 10 mL of deionized water for 15 min. It was then centrifuged (Eppendorf Microcentrifuge, Germany) at 13,000 rpm for 5 min. The supernatant was taken and passed through a 0.45-micron Millipore filter. The samples were stored at 4 °C until later analysis.

2.2.4. Phenolic compounds

Total phenols (FT) content was evaluated using the Folin-Ciocalteu method described by (21) with modifications. It started with 0.1 mL of sample added with 7 mL of distilled water and 0.5 mL of 10% Folin-Ciocalteu reagent. Subsequently, the sample was incubated for 5 min and 1.5 mL of 7.5% Na_2Ca_3 were added, to finally adjust the volume to 10 mL. The final mixture was incubated for 1 h at room temperature and darkness. Absorbance was measured at 765 nm. The FTs were expressed as mg of gallic acid/L.

2.2.5. DPPH method

A 200 µL aliquot of the fermented extract was mixed with 1800 µL of the 70% methanol DPPH solution (200 µM) and incubated for 1 h at room temperature and darkness. Protein precipitation was observed, and the sample was centrifuged at 13,000 rpm (Eppendorf Microcentrifuge, Germany) for 5 min. The supernatant was taken and put in a 96-well microplate. Absorbance was read at 515 nm. Ec. (1) was used to calculate the DPPH radical scavenging (DPPH RS) (22).

$$DPPH\ RS\ (\%) = \frac{(A_0 - A_M)}{A_0}(100) \quad \textbf{(Eq. 1)}$$

Where, A_0 is the displayed absorbance of water as the control and A_M, the absorbance of the sample.

2.2.6. ABTS method

The 7 mM ABTS•+ (5 mL) was dissolved in 5 mL of 2.45 mM $K_2S_2O_8$ and incubated for 16 h until the reaction was complete. The ABTS•+ stock solution was melted in water to an absorbance of 0.70±0.02 at 734 nm. Then, 20 µL of the fermented extract was used and mixed with 180 µL of ABTS stock solution in a 96-well microplate. Absorbance was read at 734 nm. The percentage of ABTS radical scavenging (ABTS RS) was calculated using Ec. (2) (21).

$$ABTS\ RS\ (\%) = \frac{(A_0 - A_M)}{A_0}(100) \quad \textbf{(Eq. 2)}$$

Where, A_0 is the absorbance of water as the control and A_M, the absorbance of the sample.

2.2.7. *In vitro* digestion

A sample of 10 g of the fermented with 1% inoculum and different agar-agar concentrations (0, 0.5 and 1.5% w/v) was taken. We use the methodology described by (23) with modifications for the gastric and intestinal phases. Samples were taken after each phase. Antioxidant activity (DPPH and ABTS) and total phenol content were evaluated after the intestinal phase with the methods previously presented.

Gastric phase: 10 g sample were mixed with 90 mL of 2% w/v NaCl solution, and 3.2 g/L of porcine pepsin enzyme [≥250 units/mg] (EC. 3.4.23.1, Sigma Aldrich, Mexico) was added to the electrolyte solution at a concentration of 25,000 U/mL HCl was used

to adjust the pH to 2.0. Digestion was carried out at 37° C for 2 h in a shaking bath at 10 rpm.

Intestinal phase: All sample of the gastric phase was used as a sample. We adjust the pH to 7.0 with NaOH 1 N. After this, we added the pancreatin enzyme [8 x UPS] (EC. 232-468-9, Sigma Aldrich, Mexico) at a concentration of 0.25% w/v and the bile salts at 3% w/v. Digestion was carried out at 37° C for 4 h in a shaking bath at 10 rpm.

2.2.8. Enumeration of viable bacteria

The viability of bacteria was verified using the standard pour plate technique on MRS (Man Rogosa and Sharpe) agar medium added with 0.05% w/v L-cysteine [97%] (EC. 200-157-7, Sigma Aldrich, Mexico). Serial dilutions of the gastric and intestine phase were prepared in buffered peptone water pH 7. Plates were incubated in anaerobic conditions. The bacterial count was carried out after 48 h. The results are presented in CFU/g of sample.

2.3. Statistical analysis

Analysis of variance (ANOVA), with a confidence level of 95% ($p < 0.05$), was applied using Statgraphics Plus 15.11 Software (Statistical Graphics Corporation, USA). Data are presented as the mean value of three replicas with standard deviations and assessed for normality using the Kolmogorov-Smirnow test. Statistical differences between the means were detected by Tukey's multiple range test at 95% confidence level.

3. Results and discussion

3.1. Bacterial identification

After the coconut extract fermentation was ended at the final pH of 4.5±0.2, we take a sample and isolate the bacterial colonies. The results are presented in **Table 2**. All three bacteria were found in the fermented coconut extract. With this, was verified the ability of the bacteria used as inoculum to grow in the coconut extract.

Table 2. Bacteria identification by MALDI-TOF analysis.

Morphology	Identified bacteria
Circular, large, flat, and regular edge.	*Lactobacillus acidophilus* (2.463)
Circular with center point, flat, and irregular edge.	*Lactobacillus delbrueckii* (2.391)
Circular, small, transparent, ragged edge.	*Bifidobacterium animalis* (2.288)

3.2. Probiotic survival

The minimum cell count of live probiotic bacteria to generate health benefits is around 10^6-10^7 CFU/mL. Consuming high numbers of bacteria has been suggested because, during passing through the stomach and the intestine, many cells would die (24). Thus, the survival of the strains during the production and storage time of the food product is of great importance, also, an adequate number of probiotics must reach the intestine.

The survival of the inoculum probiotic bacteria after the stomach and intestine phase is presented in Table 3. The development of nondairy yogurt-type products from plant source comes with its attendant challenges such as low probiotic count. Research has been carried out using coconut extract as a raw material to make fermented yogurt-type foods (7; 8; 19). However, these have focused on analyzing the physicochemical, textural, and sensory characteristics, therefore no

reports were found on the survival of probiotics during their production process and much less after *in vitro* digestion.

In this study, was observed after the stomach and intestine phase counts above the recommendations for health benefits. Resulting the coconut extract an alternative to cow milk with high effectiveness for the development of probiotic bacteria and act as a vehicle for its consumption. No statistically significant differences ($p > 0.05$) were found in the viability of bacteria after the stomach phase between the samples with and without the addition of agar-agar. Also, amounts above 0.5% AA may not have a greater protective effect in the stomach phase, because AA could solidify faster, limiting the number of bacteria trapped and therefore protected. Due to this, the bacterial counts of the treatments with the maximum concentration of AA and its absence present similar viability values.

Sample 2 with 0.5% w/v of AA presented survival values slightly above 0% of the AA sample after the intestinal phase. This phenomenon may be due to a more obstructive effect of the networks of "suprafibres" (20) which are linear chains of agarose that arrange themselves into double helix structures that aggregate through hydrogen bonding interactions, being the probiotic cells trapped in the network and released little by little during *in vitro* digestion by the acid conditions (21).

On the other hand, AA samples showed greater viability of bacteria compared to gelatin/agar commercial sample at the end of the *in vitro* digestion. This could mean that gelatin has a lower protective impact during its passage through simulated digestion conditions compared to agar. However, further studies about the interaction mechanism are required to verify this.

Previously, reports for fermentations in peanut extract with *B. longum* growth counts around 6.96 log CFU/mL after 6 h of fermentation (22). Due to the nature of our samples, the initial growth could not be counted at the end of the 5-hour fermentation. This, according to previous reports may be due to the obstructive effect of the networks of 'suprafibres' of agarose gels (23). However, the viability of probiotics at the end of the intestinal phase is quite positive. It has been reported for whole fat coconut extract fermented with *L. bulgaricus*; and *S. thermophilus* microbial counts around 6 log CFU/mL, but adding sucrose and glucose as replacements for lactose in cow milk (24). Also, 3.0 and 2.69 log CFU/mL of survival of probiotic bacteria in a soy yogurt fermented with *S. thermophilus* and *L. bulgaricus* after an *in vitro* digestion has been reported (25).

Table 3. Viability of probiotic bacteria during *in vitro* digestion.

Sample	*In vitro* phase	Viability [CFU/g]
1 [1.5% w/v Agar]	Stomach	2.76E+07±1.9E+08^{b}
2 [0.5% w/v Agar]		5.27E+07±1.9E+07^{a}
3 [0% w/v Agar]		2.09E+08±6.2E+07^{ab}
Commercial sample		2.37E+07±5.6E+06^{a}
1 [1.5% w/v Agar]	Intestine	6.50E+07±4.0E+07^{a}
2 [0.5% w/v Agar]		1.70E+08±8.8E+07^{b}
3 [0% w/v Agar]		8.75E+07±1.5E+07^{ab}
Commercial sample		1.52E+07±4.4E+06^{a}

3.3. Total phenols and radical scavenging

It has been reported that the principal phenol compounds present in coconut oil are: caffeic acid, *p*-coumaric acid, ferulic acid, (+/-) catechins (26), vanillic acid, syringic acid and tocopherols (27). Therefore, its presence in coconut extract even after fermentation is very likely. Previous reports suggest that the identity of individual phenolic compounds is almost same in coconut oil and coconut extract even though coconut extract contains higher quantities of individual phenolic compounds (28). Also, phenols deserve an

increasing interest for their antioxidant and anti-inflammatory properties (29).

The results of total phenols are presented in Table 4. Total phenols were significantly ($p < 0.05$) higher at the end of the *in vitro* digestion in sample 3, which is the fermented coconut extract without AA. While the lowest phenol content was found in the 1.5% AA sample. This could mean that amounts of AA above 0.5% may be overprotecting the phenolic compounds even after digestion.

This phenomenon is due to the interaction and obstructive effect caused by the AA matrix. After the low pH and digestive enzymes of the *in vitro* digestion, the phenolic compounds remain attached to the matrix, resulting in overprotection, which according to previous reports, allow bioactive substances to reach the colon and be metabolized by the microbiota and generate substances that are beneficial to health (30). The coconut extract without fermentation presented 51.21±0.1 GAE/L (data not shown), while the coconut extract after a fermentation process with 0.5% inoculum 69.08±0.88 GAE/L (data not shown). These results indicate that a greater quantity of compounds is generated during fermentation.

Regarding the ABTS and DPPH radical scavenging (Table 4), statistically significant differences ($p < 0.05$) were found. Being sample 3 (without AA) the one that presented the highest values of ABTS scavenging at the end of the digestion. The lowest values were found in sample 1 (1.5% AA) and like the behavior observed for total phenols, AA may be overprotecting the antioxidants compounds. As for the radical DPPH, no statistically significant differences ($p > 0.05$) were found.

It has been reported that for gels with AA two different effects by which water molecules reduces their movement. The (i) interaction effect, where the water interacts with the hydroxyl groups of the agar macromolecules (agarose and agaropectin). Having three-point outwards and free to hydrogen bond with either neighboring helices or the solvent. Also, the (ii) obstructive effect, by the suprafibres of the agarose gel (23) that could limit the movement of large size molecules or cells in case of the probiotic bacterial.

Table 4. Total phenols, ABTS and DPPH radical scavenging at the end of the *in vitro* digestion.

Sample	Total phenols [GAE/L]	Scavenging [%]	
		ABTS	DPPH
1 [1.5% w/v Agar]	125.7±7.3^a	54.9±0.5^a	4.6±1.4^a
2 [0.5% w/v Agar]	146.9±1.9^b	67.5±1.7^b	4.4±2.3^a
3 [0% w/v Agar]	161.8±0.1^c	70.4±0.1^c	3.4±0.5^a

4. Conclusions

Sample with 0.5% of AA showed greater viability at the end of the *in vitro* digestion. However, higher amounts of AA could be overprotecting the probiotic bacteria and also causing obstructive and interaction effect with the phenolic and antioxidant compounds in the fermented coconut extract. The sample without the addition of AA showed the maximum percentage of ABTS and DPPH radical scavenging after the digestion. This could be attributed to the presence of a less complex matrix that allows its release in an easier way. Further studies on the interaction mechanisms of the matrix generated with agar and antioxidant and phenolic compounds are required to ensure they reach their site of action and provide health benefits.

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