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Antimicrobial activity of coconut oil-in-water emulsion on *Staphylococcus epidermidis* and *Escherichia coli* EPEC associated to *Candida kefyr*

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Abstract

Candida kefyr has been considered both a food-spoiling agent and a type of yeast with fermentation properties. In this study, the authors have evaluated the antimicrobial activity of a coconut oil-in-water emulsion associated to the presence of *C. kefyr*. Fresh coconut kernels were used to obtain the coconut oil-in-water emulsion, the sterile coconut oil-in-water emulsion by decantation, and the coconut oil by means of a heating process. Commercial virgin coconut oil was also used. Agar diffusion, minimal inhibitory concentration and minimal bactericidal concentration (MIC/MBC) techniques were employed to evaluate antimicrobial activity against *E. coli* and *S. epidermidis*. The *C. kefyr* isolate was identified and confirmed. Coconut milk-derived fatty acids were characterized by acid index and thin layer chromatography. Scanning electronic microscopy was performed to evaluate the morphology of the microorganisms. Lipase activity of

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C. kefyr isolate was also detected. Coconut oil-in-water emulsion associated to *C. kefyr* was active against both bacteria. Thin layer chromatography confirmed the presence of triglycerides and free fatty acids. The acid index showed higher acidity potential for the coconut oil-in-water emulsion. The microscopic images showed antibacterial action through the formation of membrane holes' and demonstrated yeast shape. All the above show new potentials for *C. kefyr* and coconut oil-in-water emulsion in food technology.

Keywords: Microbiology, Food technology, Food science

1. Introduction

The coconut palm has been recognized as one of the world's most useful plants (Chan and Elevitch, 2006). Its products have received attention of the scientific community because water, milk, and oil all have nutritional and medicinal properties (Almeida et al., 2006; Enig, 2010). The benefits reported for coconut oil are mainly related to antibacterial properties associated to the presence of fatty acids (Kabara, 2000; Dufour et al., 2007). Additionally, coconut oil fermentation has been considered in food and pharmaceutical industries (Khoramnia et al., 2013). However, although health and manufacturing benefits are attributed to coconut oil, little is known about other coconut milk derivatives such as emulsions.

Microorganisms play an important role in physical and chemical conditions of oleaginous or emulsion products (Morais, 2009). Due to physicochemical characteristics such as water activity (a_w) and pH, fresh food offers favorable environment to yeast and molds to grow (Garnier et al., 2016). This is specially increased when microorganisms are already present *in natura* sources or through the improper handling of food (Okpokwasili and Molokwu, 1996; Cappelletti et al., 2015). In order to prolong shelf life for example, commercial coconut water will undergo heat treatments, which destroy naturally occurring microorganisms (Reddy and Lakshmi, 2014).

Coconut oil production methods may also interfere on the final product quality, as well as on its shelf-life. The cold pressing method for example, favors the growth of microorganisms. In this method, the coconut oil is obtained by squeezing the fresh kernel, which enables the production of coconut milk emulsion by decantation (Dia et al., 2005). Through this method, the humidity and nutrients will favor the biocatalysis function of yeasts (Verallo-Rowell et al., 2008; Nevin and Rajamohan, 2009).

Coconut oil-in-water emulsion is an unstable solution formed by aqueous extract of coconut solid endosperm, which is used as a cooking ingredient (Tangsuphoom and Coupland, 2009). Due to its instability, the addition of bio surfactants to coconut milk has been investigated. In this context, the enzymatic production of sugar esters

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using *Candida antarctica* lipase type B immobilized in chitosan for support can be a good alternative for the stabilization of this emulsion (Sampaio Neta et al., 2012) but according to the present study, there are no reports of complementary studies verifying whether the enzymatic process in coconut milk emulsion confers antimicrobial activity.

Candida kefyr (Beij.) (teleomorph - *Kluyveromyces marxianus*) has been suggested for beneficial use in foods as a fermentative microorganism (Bourdichon et al., 2012), playing an important role in emulsions of milk origin (Lachance, 2007; Pitt and Hocking, 2009). Nevertheless, there is a lack of information on the influence of this yeast effect on coconut-derived products. In addition, *Staphylococcus* species and *E. coli* have been considered important micro-organism related to food-borne disease (Kadariya et al., 2014; Lee et al., 2016; Møller et al., 2018). Hence, the objective of this work is to present the *in vitro* antibacterial activity of the coconut oil-in-water emulsion associated with the presence of *Candida kefyr*.

2. Material and methods

2.1. Coconut milk emulsion and oil production

Fresh coconut (*Cocos nucifera* L.) fruits characterized by irregular brown spots on the exocarp have been selected for this study. The coconut fruits were collected from the coconut palm tree farm in the Itabela district, Monte Pascoal, state of Bahia, Brazil and in public areas of Olivença, in the municipality of Ilhéus, Bahia, Brazil. The samples were registered under the plant collection of the State University of Santa Cruz (UESC) under voucher number HUESC20,054 and HUESC20,509 (acronyms by Thiers, 2016, continuously updated).

Coconut oil-in-water emulsion (COWE) was obtained by decantation of coconut milk as described by Hayatullina et al. (2012), with adaptations. First, the grazed coconut kernel (solid endosperm) was mixed with coconut water in a blender (Problend 4, Walita). Then, the humidified grazed mass was manually squeezed for extraction of the coconut milk. The coconut milk was submitted to decantation at room temperature for 24 hours. The two remaining phases were then separated and the lower phase (oil-in-water emulsion) was filtrated in a sterile tissue for the removal of larger fragments. The aqueous emulsion was then separated in two parts: one was called COWE, and the other was submitted to 121 °C for 15 minutes and called heated coconut oil-in-water emulsion (HCOWE). This sterilizing step was done to confirm the antimicrobial action due to the presence of *C. kefyr*. Both emulsions were distributed in aliquots of 6 mL and stored at room temperature. Also, one part of coconut milk was heated at 195 °C for 30 minutes to obtain coconut oil. The latter was called coconut oil obtained by heat extraction (HCO). The process was carried out in aseptic conditions and two independent experiments were performed.

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2.2. Identification and characterization of *Candida kefyr*

The presence of *Candida kefyr* in the COWE samples was determined by inoculating 10 μL of the solution over the Sabouraud dextrose (Merck, Pro-Analise, BR) agar surface. The solution was streaked in all directions, in a loop. After 48 hours of incubation at room temperature, the colonies were analyzed and the microculture technique was carried out to identify yeast genus and observe the pseudohyphe formation at 400x magnification. The Vitek 2 (Biomérieux, Brazil AS) automated system was used to confirm the yeast species. The sample received the identification number of 6057315063047100. Briefly, this system performs micro-organisms identification by continuously monitoring the growth and activity of the micro-organisms within the wells of the charts. The optical transmission system uses visible light to directly measure the growth of microorganisms at 15 minute intervals based on an initial reading prior to a significant microbial growth. The process was repeated 3 times. Auxanogram (C and N medium; raffinose, xylose, melybiose, and inositol) and zymogram (sucrose, lactose, galactose, trealose, maltose, and dextrose) were also performed to identify the yeast on the COWE solution. After 24−48 hours of incubation at 35−37 °C, the presence of gas on tubes or cloudy halo formation on carbohydrates, the peptone or potassium nitrate sites in the agar were observed.

2.3. Antibacterial activity

Staphylococcus epidermidis INCQS 00016 (ATCC 12228) and *Escherichia coli* EPEC INCQS 00182 (CDC 086H35) were obtained from the collection of microorganisms from the Reference Collection of Microorganisms on Health Surveillance (CMRVS, FIOCRUZ-INCQS), Rio de Janeiro, Brazil. Both bacteria were maintained in nutrient agar (NA; Kasvi, Liofilchem, BR) at 4 °C until use. An inoculum of each microorganism at 0.5 McFarland scale was obtained using 2.0 mL of 0.9% NaCl solution or brain-heart infusion broth (BHI; Acumedia, Neogen, Michigan) to perform the antibacterial test. The inoculum was incubated at 37 °C from 30 minutes to 2 hours prior to the tests prior broth dilution tests to adjust the inoculum to 0.5 McFarland scale.

Agar diffusion (AD), and the minimal inhibitory concentration and minimal bactericidal concentration (MIC/MBC) techniques were used for the antibacterial analysis. The AD was carried out following the M100-S22 Clinical and Laboratory Standards Institute (CLSI, 2012), with modifications. In brief, after spreading the inoculum all over the plate with a sterile swab, 6 mm diameter wells were made in the agar. The samples (50 μL) were placed in the wells and the plates were incubated in a humidified chamber, at 37 °C for 24 hours. After the incubation period, the halo diameter was measured and expressed in mm. Following the criteria established by Almeida et al. (2012), the samples producing halos >9.0 mm were considered inhibitors. Controls consisted of chloramphenicol (50 μg/mL), NaCl 0.9%, BHI,

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heat-extracted coconut oil, and commercial virgin coconut oil. The experiments were performed in triplicate and repeated three times.

In order to verify the antimicrobial action due to the presence of *C. kefyr*, MIC and MBC were performed using COWE at 99 %, 49.5 %, 29.5 %, 12.3 %, 6.18 %, 3.09 % and 1.54 % (v/v). First, the samples were solubilized with a solution containing 1% of Tween 80. Since HCOWE, HCO and VCO did not have antimicrobial action through agar diffusion nor detectable presence of *C. kefyr*, MIC/MBC were not performed for these samples. Next, 90 μL of each dilution and 10 μL of bacterial suspension were distributed in the microplate. The microplates were then incubated in a humidified chamber at 37 °C for 24 hours. The experiments were performed in triplicate and repeated at least three times. Controls consisted of chloramphenicol (50 μg/mL), 1% Tween 80 solution and BHI. After the incubation period, 10 μL of each well were transferred to Petri dishes containing nutrient agar to verify the growth of colonies.

2.4. Micro-organisms morphology analysis

The effect of COWE on bacteria morphology and morphologic aspects of the yeast were examined by scanning electronic microscopy. The technique of Tangwatcharin and Khopaibool (2012) was used for this purpose, with modifications. In brief, 1 mL of COWE at 29.5% and 49.5% containing bacterial inoculum or bacterial suspension (control) in the same concentration used for the MIC technique were incubated for 24 hours at 37 °C. After the incubation period, the samples were centrifuged at 16,000*g* for 10 minutes. The supernatant was discharged and the pellets were immersed in 200 μL of 2.5% Glutaraldehyde in 0.1 M Sodium Cacodylate buffer overnight. After that, the pellets were immersed for 10 minutes in 0.1 M (pH 7.1−7.2) Sodium cacodylate buffer. This process was repeated three times. The samples were further dehydrated in a grade series of ethanol (50%, 60%, 70%, 80%, 90% (1x), and 100% (2x) for 10 minutes each), dried in critical point for 2 hours, and sputtered with gold. The samples were visualized in scanning electron microscope (Quanta 250; FEI Company) at 30'000x magnification.

2.5. Physicochemical analysis of coconut oil-in-water emulsion

2.5.1. Acidity index

The acidity index (AI) was determined based on the methodology described by Kardash and Tur'yan (2005). For that purpose, 1.0 g of COWE was added to a 25 mL solution of ether/ethanol (2:1) to which 2 drops of phenolphthalein solution at 10 mg/mL in ethanol were instilled. Titration was done with 0.01056 mol/L NaOH standardized with Potassium hydrogen phthalate (KHP) and results were

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expressed in mg of KOH/g. After AI determination, the COWE was tested for antibacterial activity by the AD technique.

2.5.2. Thin layer chromatography of coconut oil-in-water emulsion

Thin layer chromatography (TLC) was performed with 16 × 16 cm aluminum plates covered with silica gel 60 (GF Merck) sprayed with 250 mg/mL Boric acid solution and incubated at 110 °C for 2 hours. COWE was lyophilized and suspended in chloroform prior to plate elution. The virgin oil and the heat-extracted oil were diluted in chloroform. Elution was carried out with 28.2 mL chloroform and 1.2 mL acetone and visualization was done by spreading 20% sulfuric acid solution over the plates, followed by heating at 100 °C for 5 minutes. Color aspect of spots and retention factor (R_f) were obtained for comparison with the existing standard monoglycerides (monoolein and monoheptadecanoin), diglyceride (diolein and dinonadecanoin), fatty acid (oleic acid) and triglyceride (tricaprin and triolein). The methodology followed the method by Thomas et al. (1965), with adaptations.

2.6. Lipase activity

In order to verify whether the antimicrobial action was related to the enzymatic function of *C. kefyr* yeast grown in the coconut oil-in-water emulsion, the yeast was cultivated in yeast extract (20 g/L) and sucrose (30 g/L) medium for 120 hours following the methodology by Oliveira et al. (2014). The first technique was performed using a 50 mL mixture containing ammonium sulfate (15 g/L), magnesium sulfate (0.15 g/L), yeast extract (10 g/L), Tween 80 (8 g/L), glycerol (15 g/L) and soya oil (30 g/L) in 25 mmol/L phosphate buffer, pH 6.5 was prepared for the addition of 107 cells/mL of *C. kefyr* (Winkler and Stuckmann, 1979), and the lipolytic activity was quantified by time of fermentation (72, 96 and 120 hours). The hydrolysis of p-nitrophenyllaurate (p-NFL) solution (560 μM) in potassium phosphate buffer (50 mM, pH 7.0) was also performed. Lipase activity was expressed in U/mL/min. One unit is defined as the amount of enzyme which releases 1 mmol of p-nitrophenol per minute at pH 7.0 and 37 °C (Carvalho et al., 2017).

3. Results

The amount of 4.2 L of coconut milk were used from 5.6 Kg of grazed coconut kernel to produce coconut oil-in-water emulsion and coconut oil (by heat extraction). The process yielded 3 L of COWE and 150 mL of HCO. The COWE had a cloudy appearance and rancid odor, whereas the HCO showed translucent yellow color, oily appearance and sweet odor.

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After two days in the SDA medium, it was possible to observe glossy, cream-colored colonies (Fig. 1A). Using the microculture technique, the single cells appeared elongated or oval with intracytoplasmic structures and no hypha formation was visible, which was suggestive of Candida growth (Fig. 1B and C). Zymogram and auxanogram showed sucrose, lactose, galactose and dextrose fermentation and assimilation. In addition, peptone was used as a source of nitrogen (Fig. 2). The automated system confirmed the presence of Candida kefyr in the analyzed sample.

Through agar diffusion and MIC/MBC techniques, it was possible to observe the antibacterial activity of COWE, being remarkable the bactericidal effect against *S. epidermidis* and *E. coli* at ≥29,5 % (Table 1; Figs. 3 and 4). It is worth noting that the sterilizing process by heating (HCOWE and HCO) observed in this work reinforces the antimicrobial activity associated to the presence of *C. kefyr*.

Through scanning electronic microscopy, it was possible to visualize well-defined, normal forms on pure bacteria inoculum. *S. epidermidis* presented a coccoid shape and *E. coli* EPEC a rod-shape. However, when both bacteria were submitted to COWE at 29.5% and 49.5%, they presented irregular forms and visible damage in their wall. Also, it was possible to visualize holes in the bacteria walls, as well as certain structures resembling a spider net surrounding bacterium which seemed to represent bacterial wall disintegration (Fig. 5).

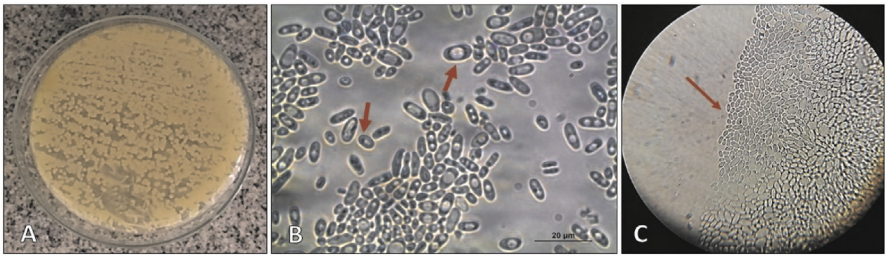

Fig. 1. Macromorphology (A) and micromorphology (B) evaluation for coconut oil-in-water emulsion yeast isolate. Red arrows indicate intracytoplasmic structures, 400x; C) Microculture for yeast detection. Red arrow indicates absence of pseudohypha, 400x.

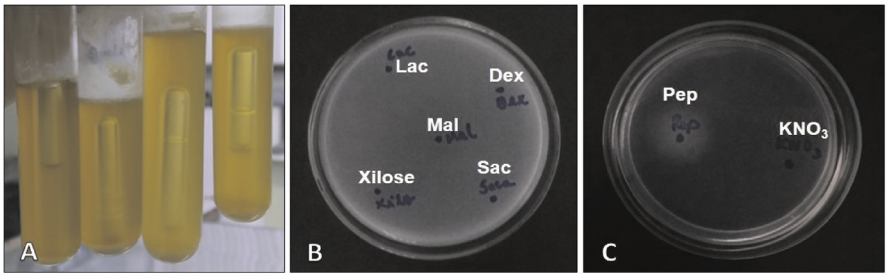

Fig. 2. Zymogram and auxanogram. A) Gas formation; B) Carbohydrate assimilation; C) Peptone assimilation. Lac – Lactose; Dex – Dextrose; Mal – Maltose; Xilo – Xilose; Sac – Sucrose; Pep – Peptona; KNO_3 – Potassium nitrate.

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Table 1. Antibacterial activity of coconut milk-derived products against *S. epidermidis* and *E. coli* EPEC through agar diffusion (AD), minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) techniques.

	***S. epidermidis* ATCC 12228**			***E. coli* EPEC CDC 086H35**		
	AD	**MIC**	**MBC**	**AD**	**MIC**	**MBC**
	mm	%	%	**Mm**	%	%
CMAE	17 ± 4.6	≥29.5	≥29.5	16 ± 3.5	≥29.5	≥29.5
HCMAE	-	nd	nd	-	nd	nd
HCO	-	nd	-	Nd	-	nd
VCO	-	nd	-	Nd	-	nd
Chlo (50 μg mL-1)	27 ± 2.6	N	N	27.7 ± 1.2	N	N
Tween 80 (1%)	-	R	R	-	R	R

Legend: R- Resistant; S- Susceptible; - no halo formation. Values represent averages and standard deviations of three independent experiments. CMAE - coconut milk aqueous emulsion; HCMAE - heated coconut milk aqueous emulsion; HCO — coconut oil obtained by heat extraction; VCO — virgin coconut oil; Chlo — Chloramphenicol; N — no bacterial growth; nd — not done.

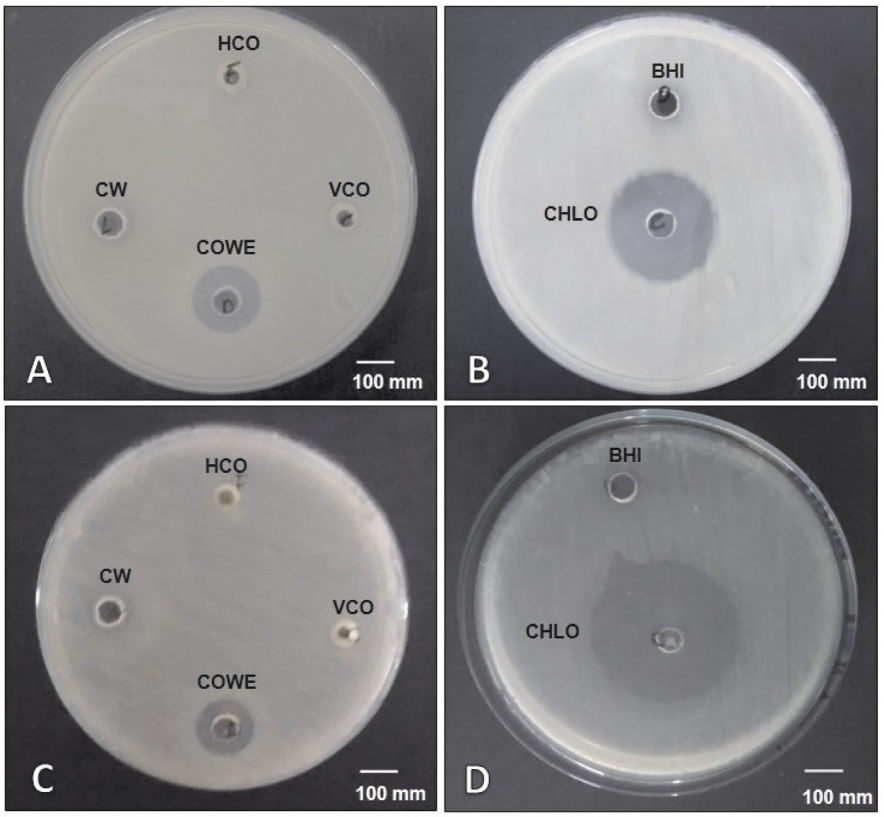

Fig. 3. Antibacterial activity of coconut oil-in-water emulsion associated to *Candida kefyr* on *S. epidermidis* (A and B) and *E. coli* EPEC (C and D) by agar diffusion technique. COWE - coconut oil-in-water emulsion; HCOWE - heated coconut oil-in-water emulsion; HCO - coconut oil obtained by heat extraction; VCO - virgin coconut oil; BHI - brain heart infusion; Chlo - Chloramphenicol (50 μg/mL).

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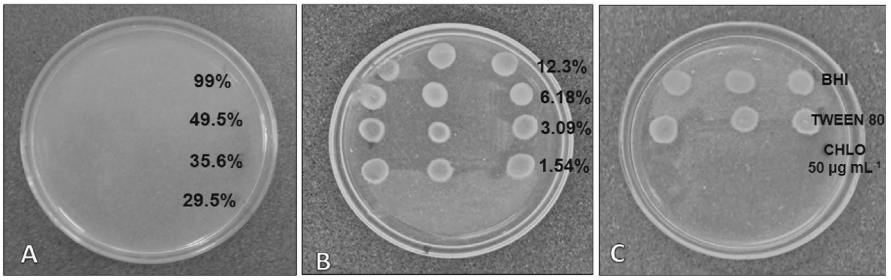

Fig. 4. Minimal bactericidal concentration of Coconut oil-in-water emulsion (COWE) associated to *Candida kefyr* against *S. aureus* and *E. coli* EPEC. A) Bactericidal effect of COWE at ≥ 29.5%; B) Bacterial growth after treatment with COWE at ≤12.3% dilution; C) Controls: brain heart infusion only (BHI), Tween 80 at 1% and Chloramphenicol 50 µg/mL (CHLO).

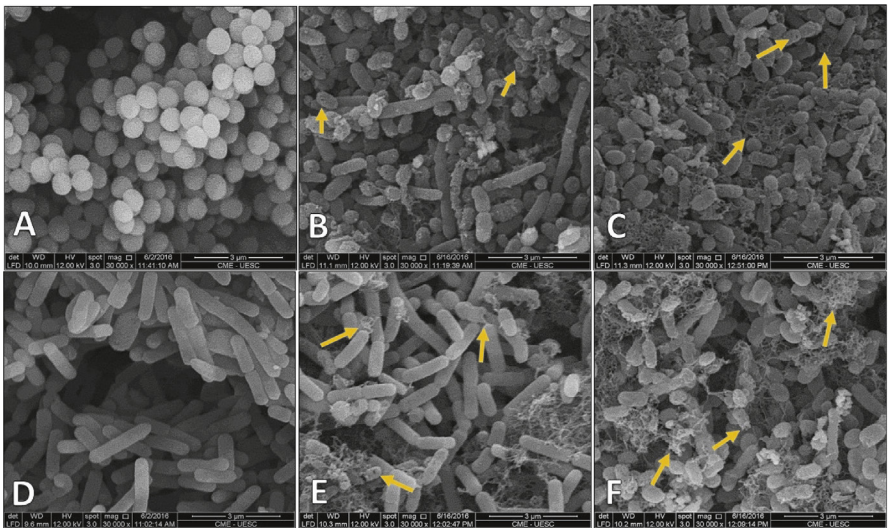

Fig. 5. Bacterial morphologic evaluation through scanning electron microscopic. *S. epiderdimis* (A, B and C) and *E. coli* EPEC (D, E and F) treated with coconut oil-in-water emulsion associated to *Candida kefyr*. A, D) Micro-organisms without treatment; B, E) COWE at 29.5%; C, F) COWE at 49.5%. Yellow arrows indicate holes in the bacterial wall. 30'000 x magnification (Quanta 250; FEI Company).

The direct analysis of COWE by scanning microscopy revealed the presence of elongated or oval unicellular microorganisms with certain rough structures which seemed to be COWE-degraded compounds (Fig. 6A). The *Candida kefyr* pure culture image revealed budding sites in one pole of the microorganisms (Fig. 6B). It is worth noting that the yeast in pure culture suspension seems to have a slight modification in the format and size when compared with growth conditions of COWE.

Acidity index (AI) values were 12.56 ± 0.52, 0.95 ± 0.34 and 1.23 ± 0.64 mg KOH/g for COWE, HCO and VCO, respectively. Rf values in TLC demonstrated the presence of fat acids compatible with fat acid (oleic acid; Rf = 0,38) and triglyceride (tricaprine − Rf = 0,89; trioleine − Rf = 0,92) used as references. Fat acids' Rf were 0.43, 0.45, and 0.44 for COWE, HCO, and VCO, respectively. Triglyceride's

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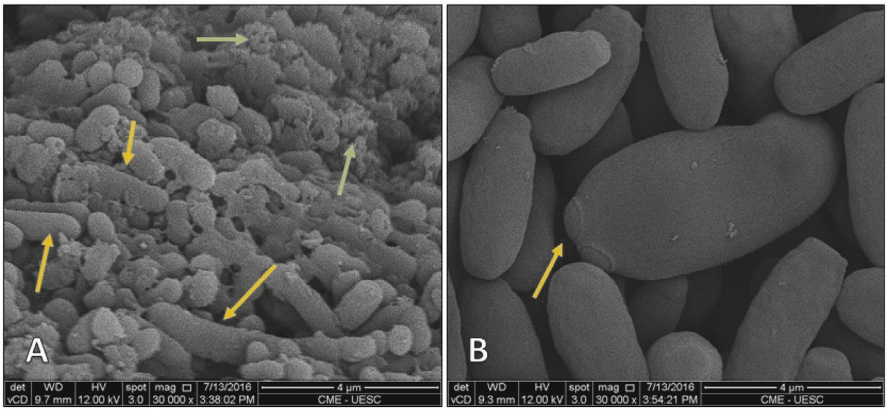

Fig. 6. Scanning electronic microscopic images of coconut oil-in-water emulsion (COWE) (A) and of yeast culture (B). A) Elongated unicellular structures in COWE. Yellow arrows indicate yeast and green arrows point to rough structures. B) Budding sites (yellow arrows). 30,000x.

Rfs were 0.87, 0.95, and 0.86 for COWE, HCO, and VCO, respectively (Fig. 7). Consequently, results confirmed the presence of free acid fats and triglycerides in all samples, however, hydrolitic degradation of triglycerides in the COWE was also seen through acidity index. It is worth noting that the not detectable mono and diglycerides spots in COWE may be due to sensibility of the technique used and they may be present in the product in small amounts.

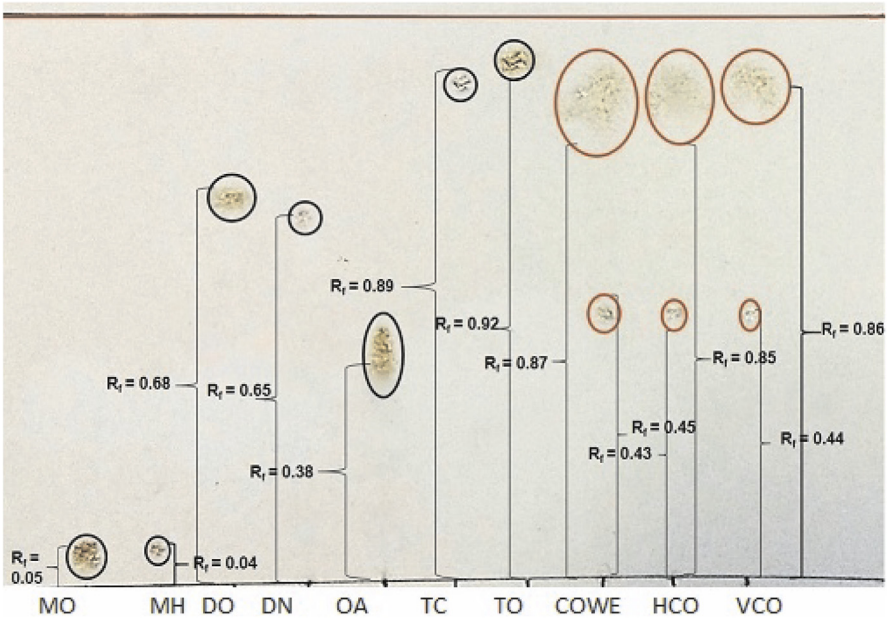

Fig. 7. Thin layer chromatography profile of coconut derivatives. Red circles represent irregular spots presented in COWE - coconut oil-in-water emulsion, HCO - coconut oil obtained by heat extraction; VCO - virgin coconut oil; Black circles represent standards. Red line — upper limit of running phase. Legend: MO - Monoolein; MH - Monoheptadecanoin; DO - Diolein; DN - Dinonadecanoin; OA - Oleic acid; TC - Tricaprin; TO - Triolein.

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To associate the triglyceride degradation to *C. kefyr* presence, the micro-organism isolated was submitted to lipase activity test. Results revealed lipase activity, being 0.179 U mL-1 min-1 at 96 hours the maximum production of lipolytic enzymes by *C. kefyr* (Fig. 8).

4. Discussion

Growth factors such as humidity and nutriments are essential to the proliferation of microorganisms. The *Candida* species for example are considered lipolytic microorganisms and they can growth well in oils and emulsions (FAO, 1979). In the present study, the aqueous and acid coconut oil-in-water emulsion environments were favorable to the formation of *C. kefyr*. The identification and characterization of *C. kefyr* associated to the physicochemical analysis of COWE compared to HCOWE, COH, and CCO support this fact. The identification and characterization of *C. kefyr* associated to the physicochemical analysis of COWE when compared to HCOWE, COH, and CCO support this fact.

The naturally-occurring microorganisms or microorganism growth caused by bad storage and handling conditions play an important role on the quality of the final product (Verallo-Rowell et al., 2008; Garnier et al., 2016); conversely, the presence of good microorganisms may protect food from spoilage through biocatalytic enzymes producing fatty acids and monoglycerides (Tangwatcharin and Khopaibool, 2012). In this study, even though the presence of monoglycerides was not evident, the hydrolytic degradation of triglycerides was clearly expressed by the AI value, antimicrobial activity, and enzyme activity detected through *C. kefyr* culture.

Extracellular lipases are usually produced intracellularly during the first hours of fermentation, and are then released to the exterior during the stationary phase. Its production decreases significantly by the end of fermentation. Fermentation times between 20 and 144 hours were reported as the best times for producing lipases

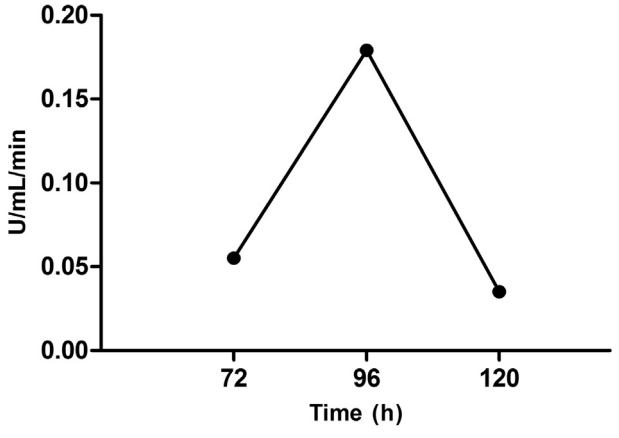

Fig. 8. Lipolytic activity (U/mL/min) of *Candida kefyr* grown by liquid-state fermentation.

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with different fungi and substrates (Sharma et al., 2001; Stergiou et al., 2013). The lipase from *C. kefyr* presented a similar profile in which the optimum fermentation time was determined to be around 96 hours during the total fermentation process.

Although *C. kefyr* has been considered yeast with technological beneficial use (Bourdichon et al., 2012) this yeast has also been considered a food spoiler (Pavlovic et al., 2014). In this study, this ambiguous function of *C. kefyr* was evident. The *C. kefyr* isolated herein simultaneously provided an antimicrobial activity to coconut oil-in-water emulsion, it equally worked as a fermentative microorganism giving undesired organoleptic characteristics which contraindicate consumption.

The microscopic images and minimal bactericidal concentration results showed herein suggest antimicrobial action of the coconut product associated to the lipolytic potential of *C. kefyr*. Thormar and Hilmarsson (2007) and Dufour et al. (2007) stated that fatty acids such as monoglycerides can cause bacterial cell wall disintegration, which in turn can interfere on nutrient transport between intra and extracellular mediums. Moreover, the effect of lauric acid and monolaurin on *S. aureus* (Tangwatcharin and Khopaibool, 2012) and *E. coli* (Kim and Rhee, 2016) has been demonstrated.

It is worth noting that the antimicrobial action of coconut oil is controversial. Bontempo (2008) stated that lauric acids are easily metabolized and they do not need to be digested by enzymes to form monolaurin, to which the antimicrobial activity is attributed. However, *in vivo* studies support the idea that antimicrobial activity of coconut oil triglycerides relies on pancreatic enzymes (Manohar et al., 2013). In the present study, the lack of antimicrobial activity of commercial coconut oil, HCOWE, and HCO in opposition to the antimicrobial activity of COWE associated to *C. kefyr* corroborate the idea of catalytic need in the antimicrobial properties of coconut milk-derived products.

5. Conclusion

The results presented here show important perspective regarding food technology: the potential of *C. kefyr* to produce antibacterial metabolites using as substrate coconut oil-in-water emulsion.

Declarations

Author contribution statement

Susy Ferraz Oliveira, Ivon Pinheiro Lôbo, João Luciano Andrioli, Camila Pacheco S. M. da Mata: Conceived and designed the experiments; Performed the

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The authors declare no conflict of interest.

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