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New cocoa pulp-based kefir beverages: Microbiological, chemical composition and sensory analysis

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ABSTRACT

The aim of the present work was to evaluate the use of the kefir grains as a starter culture for new cocoa (*Theobroma cacao* L.) beverages. Fermentation was performed by inoculating kefir grains in cocoa pulp. Flasks containing kefir grains and different substrates were statically incubated at 10 and 25 °C for 48 and 72 h. The microbiota of Brazilian kefir grains and kefir cocoa beverages was characterized using molecular techniques. Denaturing gradient gel electrophoresis displayed communities included yeasts: *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Kazachstania unispora*, and bacteria: *Lactobacillus kefiranofaciens* subsp. *kefirgranum*, *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, and a bacterium related to the genus *Acetobacter*. A microbial steady structure was detected in the analyzed kefir cocoa beverages and kefir grains. This robustness is determinant for future implementation of cocoa-based kefir beverages. The lactic, acetic, malic, propionic and citric acid contents increased during the 72 h of fermentation process in kefir beverages, reaching maximum value of ~5.55 g L^{-1}, ~1.0 g L^{-1}, ~0.3 g L^{-1}, ~1.0 g L^{-1} and ~3.0 g L^{-1} respectively. Oxalic, tartaric, butyric acids and glycerol were detected in similar concentrations. These compounds were found during the 48 and 72 h fermentation period at 10 and 25 °C in low concentration of ~1.4 g L^{-1}. Methanol also was detected in low concentration of ~0.8 g L^{-1}. The beverages fermented at 10 °C during 48 and 72 h produce lower amounts of ethanol ~4.5 g L^{-1} (0.36% v/v). These beverages had the greater acceptance (92% and 100% of the panelist, respectively) based on taste, odor, and appearance of the beverages. The best acceptance may be due to low acidity/alcoholic concentration in the beverages. In this study it was possible to produce alcoholic kefir beverages (ethanol concentration of, ~45.0 g L^{-1} (3.6% v/v)) with cocoa pulp fermentation for a period of 48 and 72 h at 25 °C. These beverages had acceptance by 80% of the panelist. This study is the first to report the alcoholic kefir beverage production from cocoa. Based on the chemical characteristics and acceptance in the sensory analysis, these results open up perspectives for this innovative application of kefir grains for developing cocoa pulp-based beverages.

1. Introduction

Cocoa (*Theobroma cacao* L.) beverages were made before 1000 B.C., extending the confirmed use of cocoa in Mesoamerica back at least 500 years. The raw cocoa, after processing is marketed mainly as chocolate. The quantity of pulp is crucial in affecting the efficiency and quality of fermentation. The excess pulp can also be sold as a high value commodity. The cocoa pulp contains 82–87% water, 10–15% sugar (60% is sucrose and 39% a mixture of glucose and fructose), 2–3% pentoses, 1–3% citric acid, and 1–1.5% pectin. Proteins, amino acids, vitamins (mainly vitamin C), and minerals are also present, being a rich medium for microbial growth (Dias, Schwan, Carlos, & Lima, 2003; Schwan, 1998; Schwan & Wheals, 2004). The pulp can be used to make jam, jelly, juice, which can be used to do fermented beverages, like cocoa wine, and other products (Duarte et al., 2010).

Kefir originated from the Caucasus Mountains and consisted of white or yellow irregular granules of protein and a polysaccharide matrix that is kefiran, produced by lactic acid bacteria. The symbiotic microbiota in kefir depends on their source and geographic region (Grønnevik, Falstad, & Narvhus, 2011; Miguel, Cardoso, Magalhães, & Schwan, 2011; Silva, Rodrigues, Filho, & Lima, 2009; Zhou, Liu, Jiang, & Dong, 2009). Kefir grains contain lactic acid bacteria (LAB) including *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Streptococcus* and yeasts (*Kluyveromyces*, *Candida* and *Saccharomyces*). The bacteria and yeast are surrounded by the kefiran matrix, which is a water-soluble branched glucogalactan (Gulitz, Stadie, Wenning, Ehrmann, & Vogel, 2011; Magalhães, Pereira, Dias, & Schwan, 2010). The

Abbreviations: CK beverage, Cocoa kefir beverage.

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fermentation of substrate makes a beverage with acidic taste, refreshing, slightly carbonated, low alcoholic and acetic content (Grønnevik et al., 2011; Miguel et al., 2011). It has been suggested that kefir has properties as a health-improving food. Several of the different bacteria and yeasts that can be found in kefir are recognized as probiotics (Latorre-García, Castillo-Agudo, & Polaina, 2006; LeBlanc, Matar, Farnworth, & Perdigon, 2006; Zhou et al., 2009). The kefir grains can be applied to ferment any kind of milk, cheese-whey or fruit juice, molasses or sugary solution, when it is named as sugary kefir, water kefir or tibico (tibico's tepache) (Koutinas et al., 2009; Magalhães, Pereira, Dias, et al., 2010).

Over the years, new and diverse methods for processing fruits have been studied in an effort to minimize production losses, increasing farmers' income, and to introduce new products to the market (Duarte et al., 2010). Several works have reported the use of fruit in producing fruit wine (Ayala-Zavala et al., 2011; Dias, Schwan, Freire, & Serôdio, 2007; Dias et al., 2003; Duarte et al., 2010). The development of fruit juice-based fermented beverage with kefir may be perceived by consumers as healthy. Therefore the aim of this paper was investigate the effect of cocoa pulp in sugary kefir fermentation and elucidate the stability, organization and identification of the dominant microbiota present in Brazilian kefir grains and correspondent beverages. Evaluation of biochemical changes, organic acids and alcohol production during fermentation process and sensory analysis of the new cocoa kefir beverage (CK beverage) was performed.

2. Material and methods

2.1. Cocoa pulp must preparation

The cocoa pods were obtained from Bahia State in the Northeast region of Brazil. The fruits were washed and broken up to extract the seeds and processed in the CEPEC/CEPLAC (Cocoa Research Centre, Itabuna, BA, Brazil). The pulp was extracted using an automatic depulping machine (ITAMETAL 0.5 DS, Itabuna, BA, Brazil).

To prepare the fermenting must, cocoa pulp was defrosted at room temperature. The cocoa pulp had an average of 13.1°Brix (°B) and pH 3.8 and was diluted in water to reduce the turbidity and adjust the soluble solids to 12°B (1:1.5 v/v). Cocoa must and control were pasteurized at 72 °C/15 min under fluent steam.

2.2. Kefir grains and inoculum preparation

Brazilian kefir grains (Stock-Culture of the Microbiology Laboratory of the Federal University of Lavras, Brazil) were used in the experiments. The inoculum was prepared by kefir grains cultivating in 12°B brown sugary solution, renewed daily, for duration of 7 days. After this time, the grains were washed with sterile distilled water and subsequently, the grains (25.5 g) were inoculated in 225 mL of 12°B fermentation substratum. The fermentation processes were developed in static conditions at 10 and 25 °C for 48 and 72 h. The static condition and temperature of 25 °C are normally used in kefir fermentation (Beshkova, Simova, Frengova, Simov, & Dimitrov, 2003; Magalhães, Dias, Pereira, Campos, et al., 2011; Magalhães, Dias, Pereira, Oliveira, et al., 2011; Magalhães, Dragone, et al., 2011; Magalhães, Pereira, Nicolau, et al., 2010; Zajšek & Goršek, 2010). The temperature of 10 °C was chosen randomly. The aim was to allow the microbial consortium present in kefir grains to grow at low temperature and analyze the differences in chemical and microbiological compositions.

Samples of the beverage were aseptically taken after the beginning and at the end of each fermentation process. Chemical and microbiological analyzes were done in all three batches. The kefir grains were recovered (filtered out) in the end of fermentation process and the beverages were evaluated by sensorial analysis.

2.3. DNA extraction and PCR–DGGE analysis

Kefir grains and fermented product, collected at the end of fermentations, were frozen at the time of sampling and stored at −20 °C. Samples of the grains used as inoculum were also collected.

Approximately 1.5 mL of each liquid sample (i.e. beverage) was centrifuged at 13,000 *g* for 5 min five times (for more and better biomass concentration). Pellets were re-suspended in 400 μL of sterile demineralized water. Each sample (grains and beverage) was transferred into a plastic tube and was subjected to DNA extraction using a NucleoSpin Tissue kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The extracted DNA was stored at −20 °C. Genomic DNA was used as template for PCR amplification of bacterial or fungal ribosomal target regions, for denaturing gradient gel electrophoresis (DGGE) analyses. Two primer sets were used for the analysis of microbial community. Table 1 shows information about the primers and PCR and DGGE conditions. All PCRs were performed in mix (50 μL) containing: 0.625 U Taq DNA polymerase (Invitrogen, Barcelona, Spain), 2.5 μL buffer 10×, 0.1 mM dNTP, 0.21 μL of each primer, 1.5 mM $MgCl_2$ and 1 μL of extracted DNA. Aliquots (2 μL) of the amplification products were analyzed by electrophoresis on 1% agarose gels and SYBR Green (Invitrogen, Foster City, USA) staining. The size of the products was estimated using a 100-bp DNA ladder (MBI Fermentas, Vilnius, Lithuania).

The PCR products were analyzed by DGGE using a Bio-Rad DCode Universal Mutation Detection System (Bio-Rad, Richmond, CA, USA). Samples were applied to 8% (w/v) polyacrylamide gels in 0.5× TAE. Optimal separation was achieved with a 30–55% urea–formamide denaturing gradient for bacteria community and 12–60% for the yeast community (100% correspondent to 7 M urea and 40% (v/v) formamide). Gels were run according to the conditions displayed in Table 1. After electrophoresis, gels were stained with SYBR Green (Invitrogen, Foster City, USA) for 30 min and photographed with a Polaroid camera. Prominent bands were excised from the gels, re-amplified, and subjected to DGGE as previously described. The new PCR products were purified using the QIAEX® III purification kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. The DNA was re-amplified using the same PCR conditions as shown above (Table 1). The PCR products were sequenced by MACROGEN (Korea). The sequences were then compared to the GenBank database using the BLAST algorithm (National Center for Biotechnology Information, MA, USA).

2.4. Physical measurement

Analyses of pH and soluble solids were performed according to the methodology proposed by AOAC — Association of Official Analytical Chemistry (2000). The pH of beverages was measured at room temperature using a digital pH meter (Micronal, B474 model, Germany). The soluble solids were determined using a digital refractometer (ATAGO PR-1000). The results are expressed in °B.

2.5. Chemicals

Sucrose, glucose and fructose were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetic acid, lactic acid, ethanol and methanol were purchased from Merck (Darmstadt, Germany). Glycerol, oxalic, citric, tartaric, malic, succinic, propionic, and butyric acids were purchased from Fluka Analytical (Seelze, Germany).

2.6. HPLC analysis

Ethanol, glycerol, organic acids (lactic, acetic, malic, propionic, butyric, oxalic, citric and malic acids), and carbohydrates (glucose, sucrose and fructose) were quantified by high-performance liquid chromatography (HPLC). Analyses were carried out using a Shimadzu chromatograph, model LC-10 Ai (Shimadzu Corp., Japan), equipped with a dual

Table 1
DGGE–PCR primers used to detect fungal and bacterial community in CK beverages.

Primer	Sequence (5′–3′)	Community	Target	PCR	DGGE conditions	References
ITS1fGC	TCC GTA GGT GAA CCT GCG G GC clamp connected to the 5′ end of ITS1gc	Fungal	ITS region of the rDNA	Condition 1	16 h at 70 V at 60 °C	a
ITS4r	TCCTCCGCTTATTGATATGC					
338fGC	GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG GC clamp connected to the 5′ end of 338fgc	Bacterial	V3 region of the 16S rRNA gene			
518r	ATT ACC GCG GCT GCT GG					

GC clamp — CGC CCG CCG CGC GCG GCG GGC GGG GCG GG.
f — forward primer; r — reverse primer.
[a]Magalhães, Pereira, Nicolau, et al. (2010).
Condition 1 — denatured for 5 min at 95 °C. 30 cycles: denaturing at 92 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s. Final extension for 10 min at 72 °C.

detection system consisting of an Ultra Violet detector (UV) and a Refractive Index Detector (RI — 10A). A Shimadzu cation-exchange column (Shim-pack SCR-101H, 7.9 mm × 30 cm), was operated at 30 °C to sugars and ethanol and 50 °C to organic acids, using 100 mM perchloric acid as the eluent at a flow rate of 0.6 mL/min. The acids were detected via UV absorbance (210 nm), while the sugars and ethanol were detected via RI. Individual sugars, acids and alcohols were identified by comparison of their retention times with the retention times of certified standards. The quantification of alcohols, sugars and acids were performed using calibration curves obtained from standard compounds. All samples were examined in triplicate.

2.7. Sensory evaluation

The final CK beverages were evaluated in sensory test. Tasters were asked to indicate how much they liked or disliked each product on a 9-point hedonic scale (9 = like extremely; 1 = dislike extremely) based on taste, odor, and appearance of the beverages, according to the Hedonic scale (Moraes, 1993) by twenty-five untrained tasters, males and females, 25–35 years of age (students and staff of the Biology Department, Federal University of Lavras, Brazil). Randomized, refrigerated (10 °C) samples of 10 mL were served in clear, tulip-shaped glasses with a volume of 50 mL; these were marked with three digit random numbers and covered with Petri dishes.

2.8. Statistical analysis

Each fermentation was carried out in duplicate and mean values ± standard deviations are reported. The Tukey's test using Statgraphics Plus for Windows 4.1 software Statistical Graphics Corp. Software - (Free download) was performed to evaluate the statistical significance (level of $P<0.05$) of differences between the beverages and to compare the means among the samples.

3. Results and discussion

3.1. Culture-independent microbiological analysis using PCR–DGGE strategy

The bacterial and yeast DGGE profiles of the four fermentations (at 10 and 25 °C for 48 and 72 h) are shown in Fig. 1a and b, respectively. No differences in community structure were found in all the fermented beverages and kefir grains, suggesting the involvement of the same group of microorganisms in the different fermentations performed. A stable population occurred throughout the fermentation. The population dynamics are related to the ability to utilize carbohydrates efficiently, efficient regulation of the redox balance, as well as tolerance toward acidification (Magalhães, Pereira, Dias, et al., 2010). As the ecological conditions remained unchanged, a stable microbiota without changes in species composition was detected. Kefir beverages constitute an environment characterized by pH about 4.5 produced by LAB – the largest group of bacteria belonging to the kefir microbiota – inhibiting the growth of other groups of microorganisms due to the antimicrobial activity of kefiran. Therefore, only few strains are highly competitive under the prevailing ecological conditions and may persist for decades in continuously propagated fermentative processes (Magalhães, Pereira, Dias, et al., 2010).

To determine the microbiota composition in grains and CK beverages a length bacterial 16S rRNA gene and fungal ITS rDNA fragments were amplified. The DGGE profiles were sequenced and the obtained sequences further compared to sequences deposited in the GenBank database using the NCBI BLAST search program (Table 2). Bacterial bands K1, K2, K3 and K4 were closest related to *Lactobacillus kefiranofaciens* subsp. *kefirgranum* (99%), *Lactobacillus plantarum* (99%), *Lactobacillus fermentum* (99%) and *L. kefiranofaciens* subsp. *kefiranofaciens* (99%), respectively, whereas K5 was affiliated to

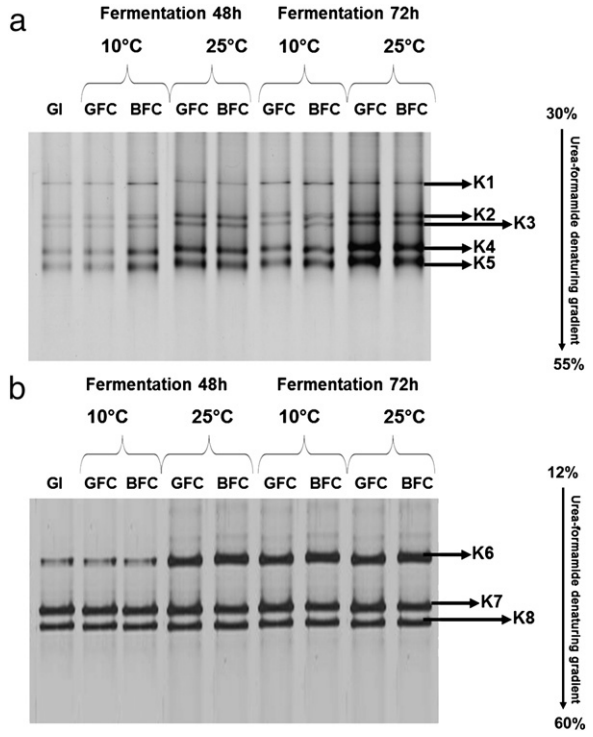

Fig. 1. Microbial DGGE profiles. (a) DGGE profiles of bacterial 16S rDNA V3 region amplified from kefir grain samples and cocoa kefir beverages. (b) DGGE profiles of fungal ITS region rDNA fragments amplified from kefir grain samples and cocoa kefir beverages. Abbreviations: GI = grain (inoculum), GFC = grain (fermentation of cocoa), BFC = beverage (fermentation of cocoa), K1 = *Lactobacillus kefiranofaciens* subsp. *kefirgranum*, K2 = *Lactobacillus plantarum*, K3 = *Lactobacillus fermentum*, K4 = *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*, K5 = *Acetobacter* sp., K6 = *Kluyveromyces marxianus*, K7 = *Saccharomyces cerevisiae*, K8 = *Kazachstania unispora*.

Table 2
Identification of bacterial and yeast bands by sequencing of portions of the 16S rRNA and ITS, respectively.

Bands	Species	GenBank accession ID	% Similarity	*E* value
K1	*Lactobacillus kefiranofaciens* subsp. *kefirgranum*	AB372208.1	99	0.0
K2	*Lactobacillus plantarum*	HQ293084.1	99	1^{-24}
K3	*Lactobacillus fermentum*	HQ293040.2	99	0.0
K4	*Lactobacillus kefiranofaciens* subsp. *kefiranofaciens*	AJ575259.1	99	0.0
K5	*Acetobacter* sp.	–	–	–
K6	*Kluyveromyces marxianus*	AF543841.1	100	0.0
K7	*Saccharomyces cerevisiae*	AM262824.1	100	0.0
K8	*Kazachstania unispora*	EU789404.1	99	$1e^{-12}$

Acetobacter genus. The band related to *Acetobacter* sp. could not be discriminated at species level according to NCBI data, showing 100% identity with more than one species of *Acetobacter* (e.g., *A. pasteurianus*, *A. lovaniensis* and *A. syzygii*). The bacterial DGGE profiles exhibited the dominant species and the intensity of each band indicated its relative abundance. *L. kefiranofaciens* subsp. *kefiranofaciens* and *Acetobacter* sp. were found at all fermentation processes and showed high intensity bands, indicating the bacterial dominant species throughout the fermentation processes. *L. kefiranofaciens* isolated from kefir is a heterofermentative bacterium that produces ethanol, acetic acid, carbon dioxide, diacetyl, acetoin, 2-3-butanediol and formate, in addition to lactic acid, and has also been reported as a kefiran (exopolysaccharide) producer in kefir (Chen, Hsiao, Hong, Dai, & Chen, 2012). *L. plantarum* is one of the most widely used lactic acid bacteria, showing a homofermentative metabolism, moderate acid tolerance, and is considered as a Generally Regarded as Safe (GRAS) organism (Brinques & Ayub, 2011).

Yeast bands (K6, K7 and K8) were closest related to *Kluyveromyces marxianus* (100%), *Saccharomyces cerevisiae* (100%) and *Kazachstania unispora* (99%), respectively (Table 2). The DGGE profiles with Eukarya-specific primer, as presented in Fig. 1b, were considerably simpler than the bacterial ecology. *S. cerevisiae* and *K. unispora* were the most prevalent species during all fermentation processes, as revealed through sequencing of the very intense DGGE band in all fermentation samples. *K. marxianus* could also be detected in all samples, but its band density was commonly weak in fermentation process of 48 h at 10 °C. Magalhães, Pereira, Nicolau, et al. (2010) reported that the presence of *Kazachstania* genus yeasts in kefir could be connected with the assimilation of some acids produced by lactic acid bacteria. *K. marxianus* shows strong activity under high acid condition, consumed part of the lactic acid, and produces ethanol and carbon dioxide (Zhou et al., 2009). The presence of *S. cerevisiae* contributes to the enhancement of the organoleptic quality of the kefir beverage, promoting a strong and typically yeasty aroma as well as its refreshing, pungent taste. This yeast also reduces the concentration of lactic acid, removes the hydrogen peroxide and produces compounds that stimulate the growth of other bacteria, thus increasing the production of exopolysaccharides (Magalhães, Pereira, Nicolau, et al., 2010).

Despite the specific differences in the microbiota of kefir grains obtained from different origins, the co-existence of a symbiotic association between lactic acid bacteria and yeasts, included in a polysaccharide–protein matrix, enabling lactic–alcoholic fermentation forms the core that characterizes the concept of kefir (Miguel, Cardoso, Lago and Schwan, 2010; Miguel et al., 2011). An important probiotic group of bacteria, i.e. *Lactobacillus* spp., is constantly found (Latorre-García et al., 2006; LeBlanc et al., 2006; Zhou et al., 2009). Being so, the probiotic properties from cocoa-based Brazilian kefir beverages found in this study are likely extensible to other fruit kefir beverages.

3.2. Physical/chemical parameters

The CK beverages were monitored during the 48 and 72 h fermentation period at 10 and 25 °C by determining the acidity. Table 3 listed the pH value changes during fermentation. The pH value decreased mainly at the first 48 h and pH value of 3.8 ± 0.04 was recorded at the end of fermentation. There was significant difference among the final pH at 10 °C (4.00 ± 0.50 (48 h) 3.94 ± 0.10 (72 h)) and 25 °C (3.84 ± 0.01 (48 h) 3.80 ± 0.04 (72 h)) in CK beverages. These results showed that the fermentation period and the temperature influenced the final acidity of CK beverages. pH values of the fermentation broth significantly influence the fermentation time of sugars and the levels of volatile compounds, reflecting possible variations in the sensory characteristics of the final product.

High performance liquid chromatography was used to analyze organic acids, ethanol and sugars produced during CK beverages. Figs. 2 and 3 show the concentration of sugars, organic acids and ethanol obtained by pulp cocoa fermentation at 10 and 25 °C for 48 and 72 h. The production processes of organic acids and alcohols were followed by the sucrose, glucose and fructose consumption in CK beverages. The total sucrose, glucose and fructose consumption were observed only after 72 h fermentation at 25 °C (Fig. 3a). The period before the 72 h of fermentation likely reflects an adaptation period of the kefir microbial community to the pulp cocoa as kefir grains are fermented in milk and brown sugar commonly. The fermented beverages at 10 °C did not show total consumption of sugars (sucrose, glucose, and fructose). This result showed that the CK beverage fermentation period and the temperature interfered with the microbiota metabolism and consequently on sugar consumption.

In the present work, the lactic acid content increased during the 72 h of fermentation process in kefir beverages, reaching maximum value of ~1.0 g L^{-1} in beverage fermented at 10 °C, and ~5.55 g L^{-1} in beverage fermented at 25 °C (Fig. 2b, 3b). The lactic acid production indicated the metabolic heterogeneity of LAB population, in which homofermentative and heterofermentative pathways are simultaneously active. The great majority of lactic-acid bacteria during fermentation utilize glucose via the Embden–Meyerhof pathway for lactic acid production. However, some species utilize glucose via the hexose monophosphate pathway producing lactic acid, ethanol, acetic acid, glycerol, mannitol, and CO_2 (Magalhães, Dias, Pereira, Campos, et al., 2011; Magalhães, Dias, Pereira, Oliveira, et al., 2011; Zajšek & Goršek, 2010).

Acetic acid was also formed during the fermentation process of CK beverages, reaching maximum value of ~0.5 g L^{-1} in 72 h of the fermentation process at 10 °C (Fig. 3b) and ~1.0 g L^{-1} in 72 h of the fermentation process at 25 °C (Fig. 2b). The acetic acid was formed probably by heterolactic bacteria and *Acetobacter*, previously identified in CK beverages and the 25 °C temperature may have contributed to the population increase of acetic acid producing microorganisms. These results are of great importance since lactic acid and acetic acid provide a pleasant taste and inhibit the development of undesirable or pathogenic microorganisms, due to the increasing acidity.

Malic, propionic and citric acids were also detected in CK beverages (Figs. 2b and 3b). Malic acid and propionic acid were detected in concentration of ~0.3 g L^{-1} and ~1.0 g L^{-1} respectively in CK beverages at 48 and 72 h of fermentation process (10 and 25 °C). Citric acid was detected in concentrations of ~3.0 g L^{-1} and ~2.5 g L^{-1} at 48 h of fermentation process (10 and 25 °C). In fermentation process at 10 °C for 72 h the citric acid concentration was almost the same for 48 h, however in fermentation process at 25 °C for 72 h the citric acid was consumed to 1.5 g L^{-1} concentration. Part of citric acid was metabolized by microorganisms as carbon and energy sources; a result of *S. cerevisiae* metabolism, that had the ability to ferment/assimilate this organic acid, causing the increase of pH value and favoring the growth of bacteria that are less acid-tolerant microorganisms (Lopandic, Zelger, Bánszky, Eliskases-Lechner, & Prillinger, 2006; Schwan & Wheals,

Table 3
Chemical compounds and pH values in CK beverages.

Temperature/time fermentation	Oxalic acid g L^{-1}	Tartaric acid g L^{-1}	Butyric acid g L^{-1}	Glycerol g L^{-1}	Methanol g L^{-1}	pH
−0 h	0.03 ± 0.01^{a}	0.04 ± 0.01^{a}	0.03 ± 0.01^{a}	n.d.	n.d.	5.30 ± 0.02^{d}
10 °C/48 h	1.38 ± 0.01^{b}	1.38 ± 0.01^{b}	1.39 ± 0.01^{b}	1.38 ± 0.01^{b}	0.79 ± 0.01^{c}	4.00 ± 0.50^{e}
10 °C/72 h	1.38 ± 0.01^{b}	1.38 ± 0.01^{b}	1.39 ± 0.01^{b}	1.38 ± 0.01^{b}	0.79 ± 0.01^{c}	3.94 ± 0.10^{e}
25 °C/48 h	1.39 ± 0.01^{b}	1.38 ± 0.01^{b}	1.41 ± 0.01^{b}	1.39 ± 0.01^{b}	0.81 ± 0.01^{c}	3.84 ± 0.01^{f}
25 °C/72 h	1.39 ± 0.01^{b}	1.38 ± 0.01^{b}	1.41 ± 0.01^{a}	1.39 ± 0.01^{b}	0.81 ± 0.01^{c}	3.80 ± 0.04^{f}

Data are mean values of duplicate ± standard deviation. Different letters indicate significant differences ($P<0.05$). n.d. = not detected.

2004). Propionic acid is an important odor-active compound in cocoa pulp and citric and malic acids are commonly found in fruit fermented beverages, acting as beverage preservatives with antimicrobial properties (Nualkaekul & Charalampopoulos, 2011).

Oxalic, tartaric, butyric acids and glycerol were detected in CK beverages at similar concentrations. These compounds were found during the 48 and 72 h fermentation period at 10 and 25 °C in low concentration of ~1.4 g L^{-1} (Table 3). The organic acids (oxalic, tartaric, butyric) produced by yeast and bacterial species, contribute to the refreshing flavor, unique aroma and texture, besides control the growth of food spoilage microorganisms (Duarte et al., 2010). The glycerol concentration detected in the CK beverages was low. This value was consistent to the below values of 10.0 g L^{-1}, suggested by Dias et al. (2007) as characteristic to confer body and texture to the beverage. Glycerol is the main secondary product in alcoholic fermentations led by *S. cerevisiae*, which was detected by sequence analysis of DGGE bands in this work.

Methanol was detected in CK beverages (during the 48 and 72 h fermentation period at 10 and 25 °C) at low concentration of ~0.8 g L^{-1} (Table 3). Methanol is an undesirable toxic alcohol in juice processing that can be produced from the hydrolysis of methyl ester groups in pectin. It occurs naturally at a low level in fresh fruit juices and most of the alcoholic beverages, at levels below 3.0 g L^{-1} (Duarte et al., 2010).

Ethanol concentration increased during the kefir fermentation process in all CK beverages, reaching maximum concentration of ~4.5 g L^{-1} (0.36% v/v) in 48 and 72 h of fermentation process at 10 °C and, ~45.0 g L^{-1} (3.6% v/v) in 48 and 72 h of fermentation

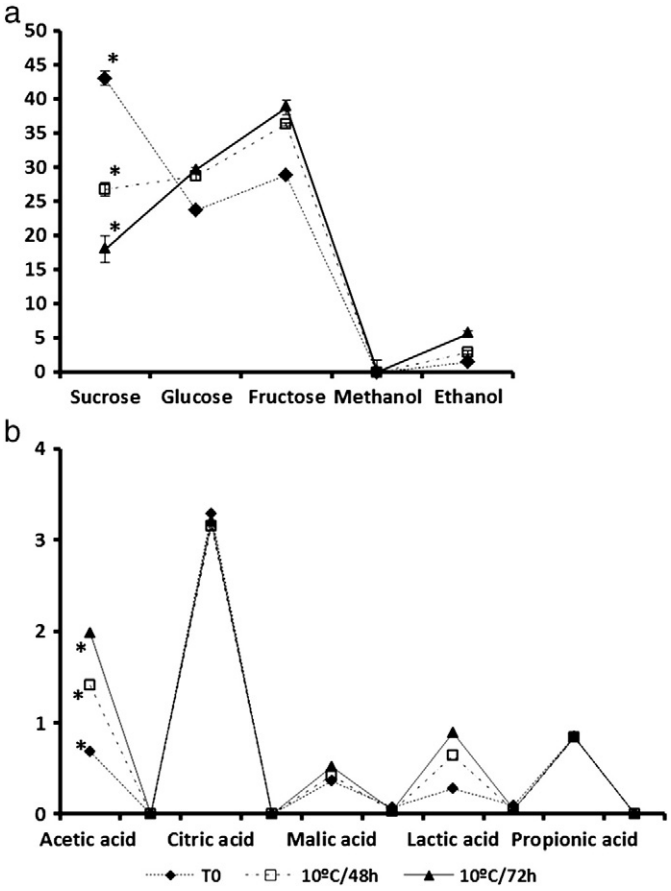

Fig. 2. Chemical parameters of the fermentation process of cocoa kefir beverages at 10 °C. (a) Sugars and alcohols. (b) Organic acids. Asterisk — Significantly different ($P<0.05$) between equal compounds.

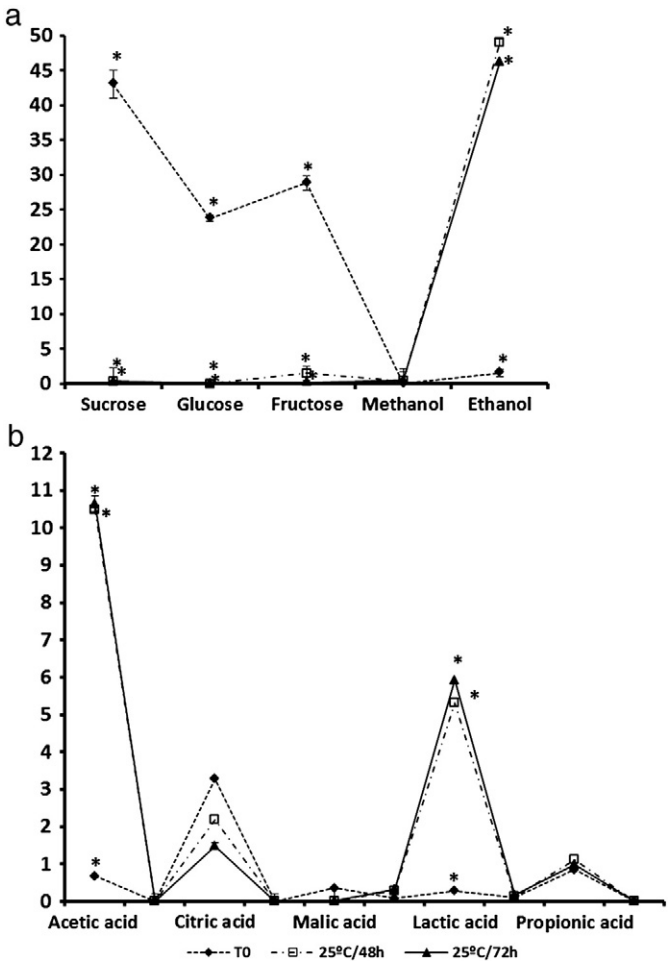

Fig. 3. Chemical parameters of the fermentation process of cocoa kefir beverages at 25 °C. (a) Sugars and alcohols. (b) Organic acids. Asterisk — Significantly different ($P<0.05$) between equal compounds.

process at 25 °C (Figs. 2, 3). The beverages fermented at 10 °C could be classified as non-alcoholics according to the Brazilian legislation (Brazil, 2009), since the ethanol content was lower than 0.5% (v/v). It has been reported that throughout the fermentation, temperature is the factor which has an important influence on the ethanol production because the lower temperatures slow down the metabolic rate of yeasts and subsequent ethanol production. Yeasts grow and proliferate rapidly and within the temperature range of 19 and 25 °C (Zajšek & Goršek, 2010). *S. cerevisiae*, previously identified in CK beverages, which exhibits strong fermentative metabolism and tolerance to ethanol, is primarily responsible for alcohol production (Pereira, Ramos, Galvão, Dias, & Schwan, 2010). However, some bacteria from the genus *Lactobacillus* also have the ability to produce ethanol, since they have alcohol dehydrogenase activity, an enzyme able to convert acetaldehyde to ethanol. *Lactobacillus* present in kefir grains grows and proliferates rapidly in temperature range higher, e.g. above 25 °C (Magalhães, Dias, Pereira, Campos, et al., 2011; Magalhães, Dias, Pereira, Oliveira, et al., 2011).

According to Beshkova et al. (2003) the content of alcohol should be enough to give kefir the flavor of a light alcoholic beverage that is typical of traditional (ancient) kefir of the Caucasus. Several studies showed low ethanol levels in kefir beverages using different substrates, such as cow's milk (10.0 g L^{-1}) (Zajšek & Goršek, 2010) and (0.05 g L^{-1}) (Magalhães, Dias, Pereira, Campos, et al., 2011; Magalhães, Dias, Pereira, Oliveira, et al., 2011), brown sugar solution (0.12 g L^{-1}) (Magalhães, Pereira, Dias, et al., 2010) and cheese whey (~11.0 g L^{-1}) (Magalhães, Dias, Pereira, Campos, et al., 2011; Magalhães, Dias, Pereira, Oliveira, et al., 2011; Magalhães, Dragone, et al., 2011; Magalhães, Pereira, Nicolau, et al., 2010). However in this study it was possible to produce alcoholic kefir beverages (ethanol ~45.0 g L^{-1}) with cocoa pulp fermentation for a period of 48/72 h at 25 °C. This result may be due to increased temperature of the fermentation process, contributing to kefir microbial increased population that produce ethanol.

3.3. Sensory analysis

Using the hedonic scale, CK beverages were subjected to sensory evaluation to assess its acceptance and preference. Beverages fermented at 10 °C during 48 and 72 h had the greater acceptance (scores 7.8 ± 1.35 and 7.28 ± 0.98, respectively) (Table 4). These beverages were the most preferred by 92% and 100% of the panelist, respectively. The beverage fermented at 25 °C with 48 and 72 h had lower acceptance (4.68 ± 2.25 and 4.56 ± 2.43, respectively) but the beverages were accepted with no extreme rejection; 20% fewer panelists accepted these beverages. The best acceptance for beverages fermented at 10 °C can be explained by the difference in metabolite compositions and acidity/alcoholic low concentration.

Table 4
Distribution of responses on hedonic scale, with resulting statistical indices for CK beverages.

Scale point description	Assigned value	Frequency of responses			
		CK beverage 10 °C/48 h	CK beverage 10 °C/72 h	CK beverage 25 °C/48 h 0	CK beverage 25 °C/72 h
Like extremely	9	3	3	0	1
Like very much	8	8	6	2	1
Like moderately	7	6	12	7	2
Like slightly	6	6	3	6	12
Neither like nor dislike	5	0	1	5	4
Dislike slightly	4	2	0	2	2
Dislike moderately	3	0	0	1	1
Dislike very much	2	0	0	2	2
Dislike extremely	1	0	0	0	0
Total responses		25	25	25	25
Mean		7.08	7.28	4.68	4.56
Standard deviation		1.35	0.98	2.25	2.43
Percentage "dislike" responses		8%	0%	20%	20%

4. Conclusion

The resulting experimental combinations of beverages enable development and deployment of effective technology-based cocoa pulp beverages, inoculated with kefir grains, in aim rationalization of production processes and expand the range of new products on market with possible nutritional value. Kefir grains were able to reduce the sucrose concentration in CK producing metabolites for good quality of the beverages. This result demonstrated the influence of fermentation period and temperature on the final CK beverage product. The beverages fermented at 10 °C during 48 and 72 h had the greater acceptance (92% and 100% of the panelist, respectively). The best acceptance may be for acidity/alcoholic low concentration in the beverages. However in this study it was possible to produce alcoholic kefir beverages with cocoa pulp fermentation for a period of 48 and 72 h at 25 °C. These beverages had acceptance by 80% of the panelist. This study is the first to report the alcoholic kefir beverage production from cocoa.

The results of this study indicate that novel beverages of acceptable organoleptic character can be produced from cocoa pulp-based fermentation by kefir grains. The proposed technology in this study is significant due to the fact that new and diverse methods for processing fruits can be used to minimize production losses, to generate more profits and to introduce new products of nutritional value to the market, including the use of probiotic kefir grains as alternative. The one key point for industrial application of the proposed technology is the promotion of fermentation by kefir of granular biomass which provides the possibility of eliminating the use of centrifugal separators that have a high energy demand and require high industrial investment.

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