Universidade
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NATÁLIA VICENTE DE REZENDE MUDENUTI

CACAU DO SUL DA BAHIA:
COMPOSTOS BIOATIVOS E METAGENÔMICA DE MICRORGANISMOS FERMENTADORES

Londrina
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Tese apresentada ao Programa de Pós-graduação em Ciência de Alimentos, nível doutorado, da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Ciência e Tecnologia de Alimentos.

Orientadora: Profª. Drª. Maria Victória Eiras Grossmann Co-orientadora: Profª. Drª. Wilma Aparecida Spinosa

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BANCA EXAMINADORA

Orientadora: Profª. Drª. Maria Victória Eiras Grosmann
Universidade Estadual de Londrina - UEL

Prof. Dr. André Luiz Buzzo Mori
Universidade Estadual de Londrina - UEL

Prof. Dr. Claudio Takeo Ueno
Universidade Tecnológica Federal do Paraná - UTFPR

Profª. Drª. Giselle Aparecida Nobre Costa
Universidade Norte do Paraná - UNOPAR

Profª. Drª. Maria Luiza Zeraik
Universidade Estadual de Londrina - UEL

Londrina, 23 de julho de 2019.

Para minha mãe,
que sempre mostrou que a Educação é o que nos define e
plantou em mim a sementinha da pesquisa.
Que me ensinou que trabalho nunca é demais e que
nossos filhos são sempre prioridade.

Para você, eu dedico.

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Nada era certo, mas parecia tão normal
Me acostumei com a incerteza ideal.
Nos faz querer o tudo, o pouco não é opção
Será surreal, ter o mundo em minhas mãos?

Tinha ao meu lado, quem soubesse me ajudar
E acreditei no que iria me tornar.
O pouco era simples, o tudo foi a opção
Será surreal, ter o mundo em minhas mãos?

Longe, alto
Cabe a cada um de nós dizer.
Onde, quando
Só cabe a cada um de nós saber.

Scalene - Surreal

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RESUMO

A crescente demanda por alimentos naturalmente ricos em compostos bioativos leva a indústria a buscar processamentos cada vez mais amenos para que se preserve o conteúdo dessas substâncias nas matérias-primas. A atividade antioxidante é um dos aspectos benéficos que mais atrai a atenção dos consumidores e os compostos fenólicos são subtâncias que atuam diretamente na interupção de reações oxidativas promovendo diversos efeitos biológicos ao consumo humano. Outro grupo químico amplamente utilizado pelo homem por sua atividade estimulante é o dos alcaloides, no qual, as metilxantinas como theobromina e cafeína são consumidas há séculos devido aos efeitos no sistema nervoso central. Ambos grupos são naturalmente presentes no cacau e em seu derivados, fazendo com que sua ação bioativa seja muito estudada e levando o consumidor a prestar mais atenção no tipo de chocolate que consome. A região sul da Bahia compõe o cenário dos produtores mundiais de cacau desde o século XVIII, após uma grande crise da atividade cacaueira encontrou na qualidade das amêndoas e no método de cultivo tradicional uma forma para reestabelecer uma identidade capaz de recolocar o produto da região no níveis das demandas internacionais. A consolidação desses esforços se deu com a criação da Indicação Geográfica de procedência. A torrefação é uma importante etapa na formação de precursores de sabor do chocolate, mas também a que mais impacta na degradação dos compostos bioativos. Com o intuito de oferecer um chocolate com o mínimo de perda dessas substâncias, produtores começaram a produção de chocolates com as amêndoas não torradas, o chocolate cru. O aumento do interesse pelo produto cru levou à disseminação do consumo das amêndoas de cacau e de sua testa também não torradas. Esse trabalho teve como objetivo avaliar o perfil fenólico e de alcalóides com sua quantificação por UHPLC-PDA e de sua atividade antioxidante pelas metodologias FRAP, DPPH, ABTS e ORAC em amostras de nibs e testa de cacau da região sul da Bahia que comercializam o produto cru e no chocolate produzido com elas. A extração dos compostos insolúveis se demonstrou crucial para quantificação de teofilina, ácido protocateuico e catequina nos chocolates, além de trigonelina nos nibs e na testa e possibilitou a constatação que a torrefação promove a liberação desses compostos presos a parede celular. Em chocolates com menor teor de cacau, a contribuição dos compostos insoluveis chegou a 40%. O chocolate cru apresentou maiores teores de compostos bioativos em geral em comparação ao chocolate tradicional, com niveis máximos de 36.812 mg g^{-1} de epicatequina, 16.416 mg g^{-1} de catequina, 40.103 mg g^{-1} de theobromina, 28.584 mg g^{-1} de cafeína e 4.465 mg g^{-1} de ácido protocatecuíco. Nos nibs e testa, a catequina foi o flavonóide de maior concentração alcançando 33.308 mg g^{-1}, enquanto a epicatequina chegou a 18.333 mg g^{-1}. As quantidades de ácido protocatecuíco chegou a 12.235 mg g^{-1} em testa de cacau. A relação entre as quantidades de cafeína e teobromina encontradas nos nibs e testa classificaram o cacau adequado para o tipo forastero. Dando sequência à caracterização da produção local, a fermentação conduzida em quatro fazendas certificadas na indicação geográfica foi caracterizada quanto aos microrganismos presentes por meio da analise metagenômica. A comunidade microbiana se demonstrou como um consórcio de leveduras (Candida, Terulaspora e Saccharomyces), bactérias lácticas (Lactobacillus, Leuconostoc e Pseudomonas) e bactérias acéticas (Acetobacter e Gluconobacter).

Palavras-chave: Indicação geográfica. Compostos fenólicos. Atividade antioxidante. Metilxantinas. Chocolate.

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ABSTRACT

The increasing demand for foods naturally rich in bioactive compounds leads the industry to seek for milder processing steps aiming to preserve these substances in the ingredients. Antioxidant activity is one of beneficial aspects which attract consumers' attention. Phenolic compounds acts directly interrupting oxidative reactions and promoting biological effects to human consumption. Another chemical group widely used by man because its stimulating activity is the alkaloids, methylxanthines such theobromine and caffeine is been consumed for centuries because of their effects on the central nervous system. Both groups are naturally present in cocoa and its derivatives making its bioactive action widely studied and prompting consumers to pay more attention to the type of chocolate they buy. Southern Bahia region composes the scenario of the world producers since the 18th century and, after a major crisis of cocoa activity, found in the quality of the beans and in the traditional harvesting method a way to reestablish an identity capable of re-locating the region's product at the level of international demands. The consolidation of these efforts occurred with the creation of the Geographical Indication of precedence. Roasting is an important step in the formation of chocolate flavor precursors but also has a great impact on the degradation of bioactive compounds. With the intention of offering a chocolate with the minimum loss of these substances, producers began to make the chocolate with unroasted beans, the raw chocolate. The increasing interest in the raw product led to the spread of consumption of cocoa nibs and husk also unroasted. The objective of this study was evaluate the phenolics and alkaloids profile quantifying with UHPLC-DAD and its antioxidant activity by total phenolic content, FRAP, DPPH, ABTS and ORAC methods in chocolate, cocoa husk and nibs produced in Southern Bahia by producers dedicated to raw products. Insoluble-bound extraction was critical to quantify theophylline, protocatechuic acid and catechin in chocolate, plus trigonelline in husk and nibs and made it possible to confirm that roasting promotes the release of these compounds from the cell wall. In lower cocoa solids content raw chocolates insoluble-bound compounds contributed until 40% of total amount. Raw chocolate presented higher levels of bioactive compounds in comparison to traditional chocolate, with higher levels reaching 36.812 mg g^{-1} of epicatechin, 16.416 mg g^{-1} of catechin, 40.103 mg g^{-1} de theobromine, 28.584 mg g^{-1} of caffeine and 4.465 mg g^{-1} de protocatechuic acid. In nibs and husk, catechin was the major flavonol found reaching 33.308 mg g^{-1}, while epicatechin presented maximum amount as 18.333 mg g^{-1}. Protocatechuic acid was 12.235 mg g^{-1} in cocoa husk. Caffeine/theobromine relation in husk and nibs properly classified the cocoa in the forastero type. Following the characterization of local production, the cocoa beans fermentation conducted on four farms certified in the geographical indication was characterized for the microorganisms present through the metagenomic analysis. The microbial community demonstrated a consortium between yeasts (*Candida, Terulaspora* and

Saccharomyces), lactic acid bacteria (*Lactobacillus, Leuconostoc* and *Pseudomonas*) and acetic acid bacteria (*Acetobacter* and *Gluconobacter*).

Key words: Geographic indication. Phenolic compounds. Antioxidant activity. Methylxanthines. Chocolate.

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LISTA DE ABREVIATURAS E SIGLAS

AAPH	2,2'-Azobis(2-amidinopropane) dihydrochloride
AAB	Acetic acid bacteria
ABICAB	Associação Brasileira das Indústrias de Cacau, Amendoim e Balas
ABTS^{TM}	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
CABRUCA	Cooperativa dos Produtores Orgânicos do Sul da Bahia
CENEX	Centro de Extensão
CEPEC	Centro de Pesquisa do Cacau
CEPLAC	Comissão Executiva do Plano da Lavoura Cacaueira
CNPC	Central Nacional dos Produtores de Cacau
DAD	Diode-array Detector
DNA	Ácido Desoxirribonucleico
DPPH	2,2-diphenyl-1-picrylhydrazyl
EMARC	Escola Média de Agropecuária da Região Cacaueira
FAO	Food and Agriculture Organization of the United Nations
FRAP	Ferric Reducing Antioxidant Potential
GAE	Gallic Acid Equivalent
GI	Geographical Indication
HDL	High-density Lipoprotein
ICB	Instituto do Cacau da Bahia
IG	Indicação Geográfica
LAB	Lactic acid bacteria
LDL	Low-density Lipoprotein
ORAC	Oxygen Radical Absorbance Capacity
rDNA	Ácido Desoxirribonucleico Ribossomal
ROS	Reactive Oxygen Species
SBC	Southern Bahia Cocoa
TPC	Total Phenolic Content
TPTZ	2,4,6-Tris(2-pyridyl)-*s*-triazine
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UHPLC	Ultra-high-performance liquid chromatography
VLDL	Very-low-density Lipoprotein

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CAPÍTULO 1

INTRODUÇÃO, REVISÃO BIBLIOGRÁFICA E OBJETIVOS

1.1 INTRODUÇÃO

Tendências globais impulsionam o desenvolvimento da indústria de alimentos que, por sua vez, busca atender as novas demandas de mercado. Duas delas vêm demonstrando grande crescimento: os alimentos funcionais e a produção *fair trade,* uma modalidade de comércio internacional que busca o estabelecimento de preços justos, padrões sociais e ambientais equilibrados nas cadeias produtivas, promovendo o encontro de produtores responsáveis com consumidores éticos.

O conceito da produção *fair trade* representa o desafio da indústria de chocolates em tornar a produção socialmente sustentável, eliminando os bolsões de pobreza que se formam nas regiões produtoras de cacau.

O sul da Bahia é composto pela Mata Atlântica, que permite que os cacaueiros cresçam à sombra de suas grandes árvores. A umidade elevada e a altitude da serra criam um agrossistema denominado *Cabruca.*

Como tentativa de recuperar uma identidade comercial, após o colapso da produção cacaueira na década de 90, a região produtora Sul da Bahia recebeu a certificação de Indicação Geográfica. Através da implantação de sistemas de controle de qualidade e das práticas de manejo tradicionais à Cabruca, os produtores vêm alcançando o reconhecimento dos consumidores nacionais e internacionais e aumentando o valor do produto, ao sair do patamar de cacau *commodity.*

Por si só, o cacau é um ingrediente que se qualifica como funcional, devido à grande quantidade de flavonóides que possui. Os flavonóides são uma subclasse dos polifenóis, compostos fitoquímicos ativos, que apresentam atividade antioxidante. Estão presentes na forma monomérica, como a epicatequina e a catequina, e na de flavonóides oligoméricos, as procianidinas (LAMBERT, 2009).

Segundo Engler et al. (2004), o benefício cardiovascular dos flavonóides vem sendo atribuído às suas propriedades antioxidantes naturais e seu papel em conservar tocoferóis em membranas. Um estudo epidemiológico conduzido por esses autores constatou que chocolates ricos em flavonóides melhoram a vasodilatação do endotélio, efeito associado ao aumento das concentrações e epicatequina no plasma.

Os compostos fenólicos são produzidos pelos vegetais, como mecanismo de defesa ao ataque de fungos e insetos, sendo encontrados aderidos às paredes celulares e insolúveis em água. Por não serem assimilados durante a digestão, são hidrolisados pelas enzimas de microrganismos do intestino, passando a ter atividade prebiótica (NACZK; SHAHIDI, 2009).

As plantas também podem produzir polifenóis como compostos de reserva de nutrientes. Para essa finalidade, são armazenados em vacúolos e se encontram na forma solúvel, sendo absorvidos no intestino delgado e atingindo as células através da circulação sanguínea.

Outro importante grupo de compostos com atividade biológica é o dos alcaloides, responsáveis pela estimulação do sistema nervoso central e de imediata ação estimulante de alerta e da memória. O cacaueiro é a planta utilizada pelo ser humano na alimentação com maior produção de teobromina e também apresenta quantidades significativas de cafeína e teofilina.

Devido a esses benefícios, o interesse em produzir chocolates com maiores concentrações de compostos bioativos é crescente, na mesma medida em que os consumidores também estão mais atentos em ingerir esse tipo de alimento.

A etapa de processamento que mais influencia na diminuição das concentrações de antioxidantes no chocolate é a torrefação das sementes. Buscando um alimento minimente processado, que mantivesse os compostos bioativos nas concentrações mais próximas do cacau *in natura*, produtores eliminaram a torrefação, obtendo assim o chocolate cru.

A semente do cacau possui uma película chamada testa, que é removida para a produção de chocolate e passou a ser estudada como ingrediente de alto potencial funcional, ao invés de ser descartada ou utilizada como adubo orgânico. Com a produção do chocolate cru, a semente do cacau, comercializada como nibs, e a testa passaram a ser disponibilizadas também nas versões cruas, sem passarem pela torra.

Os produtos de cacau cru ainda são novidade para muitos consumidores e não existem dados científicos que comprovem seus beneficios como alimento funcional e sua segurança, do ponto de vista microbiológico.

Caracterizar esses produtos é um passo que pode trazer novos alimentos funcionais para a dieta e um produto com maior valor agregado para os produtores de chocolate da região.

A primeira etapa de processamento que interfere na qualidade do cacau, a fermentação das sementes, é realizada ainda nas fazendas. Por se tratar de uma sucessão biológica natural, onde diversos agentes ambientais influenciam na fermentação, uma diversidade de espécies pode ser encontrada. Uma vez que os métodos tradicionais de cultivo microbiano recuperam apenas espécies cultiváveis e analisam membros específicos da comunidade microbiana, as técnicas tradicionais não demonstram uma diversidade confiável da microbiota do cacau.

Dessa forma, as abordagens moleculares independentes de cultura constituem uma alternativa importante aos métodos tradicionais de cultivo ao permitir o estudo de toda a diversidade microbiana. Nesse contexto, a análise metagenômica é mais nova e importante abordagem no contexto das ferramentas de análise de comunidades microbianas complexas, como a fermentação do cacau.

Tendo em vista o importante papel da etapa da fermentação na conversão dos compostos bioativos e na formação dos precursores de sabor que caracterizam o chocolate, a microbiota fermentativa da região da IG Cacau do Sul da Bahia é de grande interessa para o estabelecimento da superioridade do processo e obtenção de sementes de cacau de alto valor de mercado.

1.2 REVISÃO BIBLIOGRÁFICA

1.2.1 A evolução do setor cacaueiro no Brasil

O cacaueiro (*Theobroma cacao* L.), descrito por Lineu em 1737, pertence à ordem Malvales, família Malvaceae e gênero *Theobroma*. A árvore do cacau é uma espécie nativa da floresta tropical úmida americana, que se desenvolve em clima quente e úmido, provavelmente originária das bacias dos rios Amazonas e Orinoco. (SOUNIGO et al., 2003; HENDERSON et. al., 2007). A árvore atinge entre 5 a 10 metros de altura e os primeiros frutos são colhidos cerca de 5 anos após a plantação. O fruto tem forma oval, com 15 a 20 cm de comprimento, e cor que vai do amarelo ao vermelho, quando maduro (MARTINI, 2004; BATALHA, 2009; BECKETT, 2009).

Existem três variedades de cacau: forastero, criollo e trinitário. A variedade forastero possui cotilédones de cor púrpura, devido à presença de antocianinas e é, comercialmente, a variedade mais abundante. O cacau criollo, classificado como mais suave, possui cotilédones brancos sendo considerado de qualidade superior; entretanto, é menos vigoroso e mais vulnerável a doenças que o primeiro. A terceira variedade, Trinitário, é um híbrido entre o forastero e o criollo e seus cotilédones possuem coloração que varia do branco ao púrpuro (HANCOCK, 1994; BART-PLANGE; BARYEH, 2003). O grupo forastero corresponde a 80% da produção mundial, sendo predominante na zona cacaueira da Bahia (MARITA et al., 2001; LEMUS et al., 2002).

O uso das sementes do cacaueiro, como fonte de alimento, começou com as civilizações pré-colombianas na América Central, sendo dado como especiaria e ingrediente principal de uma bebida apimentada chamada *chacauhaal*, de *chacau*, que significa quente, e *haa*, que significa bebida. Os Incas consideravam as bebidas preparadas com o cacau como a “bebida dos deuses”, e esta associação deu origem ao nome científico, *Theobroma cacao* (LEVANON; ROSSETINI, 2005; HENDERSON et al, 2007;).

O cacau chegou à Europa como uma fruta exótica e foi elevado a alimento de luxo quando misturado ao açúcar e ao leite, sendo, então, chamado de chocolate. Com sabor e textura únicos, o refinado confeito logo ganhou popularidade, passando a ser produzido em escala industrial (BECKETT, 2009).

A introdução da cultura de cacau no Sul da Bahia data de 1746, quando sementes trazidas do Pará foram plantadas no município de Canavieiras, expandindo-se para diferentes zonas da região (CABALA-ROSAND, 1998). O clima favoreceu o plantio e as lavouras cacaueiras prosperaram na região, que ficou conhecida pelos coronéis do cacau e um sistema econômico que funcionava em torno do seu plantio, beneficiamento e comercialização (ROCHA, 2008).

A produção abundante de cacau, que primariamente era exportada, levou os irmãos Neugebauer e o sócio Gerhardt, todos imigrantes alemães, a fundar a primeira fábrica de chocolate brasileira, em Porto Alegre, em 1891. Vinte e um anos depois, o cônsul suíço Achilles Izella lançou a pedra fundamental de outra marca nacional, a Lacta. Em seguida, vieram a Kopenhagen, em 1928, e a Garoto, em 1929. Em 1959, a suíça Nestlé começou a fabricar chocolates no Brasil (ABICAB, 2019).

O cultivo do cacau requer temperatura máxima e mínima de 32 e 18°C, respectivamente, um período de seca inferior a três meses e um índice pluviométrico entre 1000 e 4000 mm ao ano. O solo deve ser profundo e fértil e a cultura é muito susceptível a pragas e fungos (CRUZ, 2012). Os principais estados produtores de cacau do Brasil: Bahia, Amazonas, Pará, Espírito Santo, Rondônia e Mato Grosso, viveram e vivem altos e baixos na produção e exportação desse produto agrícola.

No caso específico do Sul da Bahia, principal área produtora do Estado e do país, o volume de produção e prosperidade econômica levaram à criação do Instituto do Cacau da Bahia – ICB e da Comissão Executiva do Plano da Lavoura Cacaueira - CEPLAC, com todos os seus órgãos: Centro de Pesquisa do Cacau - CEPEC, Centro de Extensão - CENEX, Escola Média de Agropecuária da Região Cacaueira - EMARC; a hoje Central Nacional dos Produtores de Cacau – CNPC. A região vivenciou uma fase de prosperidade sem precedentes, que se estendeu da segunda metade da década de 1970 até meados da década de 1980, período após o qual mergulhou numa situação de grandes dificuldades (ROCHA, 2008).

Desde 1986, essa região amarga os impactos de uma longa crise de preços devido a uma superprodução mundial de cacau. Para agravar a situação, em 1989, teve início o alastramento do fungo *Crinipellis perniciosa*, causador da vassoura-de-bruxa. Com esse fato, a crise se aprofundou, os produtores de cacau se endividaram, houve abandono de plantações e aumento do desemprego rural e urbano (ROCHA, 2008).

A má qualidade do cacau produzido implicava em menores preços, e consequentemente, a renda dos que dependiam da atividade gerando bolsões de pobreza. A única saída para reverter a situação era voltar a produzir com qualidade, elevar o preço da produção e reestabelecer a imagem do cacau baiano internacionalmente. Os primeiros resultados positivos vieram do aproveitamento das características ambientais únicas que o cultivo do cacau Baiano possui, criando o sistema Cabruca.

Em 2001, foi fundada a Cooperativa dos Produtores Orgânicos do Sul da Bahia (CABRUCA), sediada em Ilhéus, com a missão de reunir agricultores cujas propriedades estejam localizadas no entorno dos remanescentes da Mata Atlântica. Atualmente, fazem parte dela mais de 40 produtores, distribuídos em 1500 hectares, com o objetivo de inovar na produção, melhorar os preços e satisfazer o mercado internacional que dá preferência a produtos que tenham tal origem (ROCHA, 2008).

Na região cacaueira da Bahia, a CABRUCA propõe-se, além de proporcionar condições para que haja um comércio justo, a "descomoditizar" o cacau, ou seja, tirá-lo do grupo das mercadorias não especializadas, produzidas em massa, cujo padrão ou referência sempre será o preço mais baixo (ROCHA, 2008).

A concretização dos esforços dos produtores em identificar a produção de cacau do sul da Bahia por sua qualidade, se deu em 24 de abril de 2018, com a aprovação da Indicação de Procedência "Sul da Bahia". A partir da regulamentação da identificação geográfica, produtores de 83 municípios situados entre os paralelos 13º03' e 18º21' sul e os meridianos 38º51' e 40º49' a oeste de Greenwich passaram a produzir amêndoas de cacau podendo receber o selo IG, certificado pelo Conselho Regulador da Associação Cacau Sul da Bahia (BRASIL, 2018).

Segundo a FAO (2018), as IG promovem interação entre produtores locais e são um caminho para criação de sistemas alimentares nutritivos e desenvolvimento sustentável para comunidades rurais ao redor do mundo. A qualidade e atributos específicos do alimento são ligados a sua origem. Em diversas partes do mundo, gerações têm construído sua identidade local através de produtos alimentícios típicos e uma paisagem específica que refletem as interações entre os recursos naturais e os sistemas produtivos. Hoje, essas ligações entre produtos, lugares e habitantes não só representam uma herança a ser preservada, mas também possuem um valor de mercado por direito próprio, uma vez que os consumidores se interessam cada vez mais pela qualidade agregada à origem geográfica e tradição.

O faturamento do mercado de chocolates no Brasil, em 2018, foi de R$ 13,3 bilhões, permanecendo em sexto lugar entre os seis países que lideram o volume de vendas de chocolate no varejo, depois de EUA, Rússia, Alemanha, Reino Unido e França. A indústria se concentra, especialmente, em São Paulo, mas há importantes fabricantes também na Bahia, Espírito Santo, Paraná e Rio Grande do Sul. Atualmente o Brasil exporta chocolates para mais de 124 países (ABICAB, 2019).

1.2.2 A fermentação do cacau e a metagenômica

Antes de chegar à produção de chocolates, a semente do cacau passa por diversas etapas de preparo nas fazendas onde este é cultivado. Logo após a colheita, os grãos são fermentados para a remoção da mucilagem que os envolve. Esse processo desenvolve componentes químicos dentro da semente, que são precursores do sabor no chocolate. Falhas nesse estagio não podem ser corrigidas com as demais etapas de processamento (BECKETT, 2009).

As sementes de cacau possuem sabor adstringente desagradável, devendo ser fermentadas para que seus compostos possam ser convertidos em precursores do sabor de chocolate, tão apreciado. Cada semente é composta de dois cotiléndoles e um embrião (radícula), rodeados por um tegumento (testa) e envoltos por uma polpa mucilaginosa branca e adocicada, que equivale a 40% do peso das sementes frescas (SCHWAN; WHEALS, 2004; LEFEBER et al., 2010).

A polpa e as sementes são estéreis quando se encontram em maturação no cacaueiro. Com a colheita e coleta das sementes ocorre a contaminação natural com a variedade de microrganismos do ambiente, muitos dos quais irão contribuir com fermentação subsequente. A difusão dos metabólitos das etapas de fermentação engatilham reações bioquímicas complexas nos cotiléndoles, que levam à morte do embrião e os tornam estáveis ao armazenamento, após a secagem (SCHWAN; WHEALS, 2004).

O processo de fermentação ocorre como uma proliferação espontânea da microbiota local; as sementes podem estar acondicionadas em pilhas, sobre o solo forrado com folhas de bananeira, cestos, caixas de madeira ou plataformas por cerca de seis dias e é a base de toda produção de chocolates e produtos à base de cacau (LEFEBER et al., 2010).

Na produção de culturas que passam por fermentação espontânea, a compreensão do complexo papel entre os diferentes grupos de microrganismos é da maior importância para o controle do processo e do seu impacto na qualidade do produto (PEREIRA; MAGALHÃES-GUEDES; SCHWAN, 2013).

Em estudo de identificação da diversidade da microbiota responsável pela fermentação das sementes de cacau, no Estado da Bahia - Brasil, e de sua distribuição durante o processo fermentativo, Pereira; Magalhães-Guedes & Schwan (2013), identificaram cepas de *Acetobacter* sp.*, Bacillus* sp.*, Bacillus lichenformis, Bacillus firmus, Frauteria aurantia, Hanseniaspora* sp.*, Lactobacillus* sp.*, Lactobacillus plantarum, Lactobacillus fermuntum, Saccharomyces cerevisiae, Tatumella ptyseos e Xanthomonas* sp.

A polpa do cacau é um rico meio de cultura microbianom constituída de aproximadamente por 84% de água, 10% de mono e dissacarídeos, 2% de pentosanas, 2% de ácido ascórbico e 1% de pectina. Proteínas, aminoácidos livres e vitaminas também estão presentes em menores concentrações. A proporção entre sacarose, glicose e frutose presentes na polpa varia de acordo com o estágio de maturação do fruto (SCHWAN; WHEALS, 2004).

Para que a fermentação seja bem sucedida, determinadas atividades microbianas particulares são exigidas. No inicio da fermentação, as leveduras são dominantes e provocam a liquefação da polpa através da despectinização. Outra atuação importante das leveduras é a produção de etanol, através da fermentação de carboidratos, sob condições anaeróbicas e acidez favorável (LEFEBER et al., 2010).

O grupo das leveduras é dominado por *S. cerevisiae* e *Hanseniaspora* sp., ocorrendo com frequência também *Pichia kudriavzevii* e *Pichia membranifaciens* (PEREIRA; MAGALHÃES-GUEDES; SCHWAN, 2013).

Conforme a polpa começa a ser drenada, o ar penetra no mosto formado criando condições ideais para o desenvolvimento de bactérias microaerófilas e ácido-tolerantes, especialmente as bactérias ácido láticas como *Lactobacillus plantarum* e *Lactobacillus fermentum* que convertem a glucose, frutose e ácido cítrico em ácido lático e/ou manitol diminuindo o pH da polpa (LEFEBER et al., 2010, PEREIRA; MAGALHÃES-GUEDES; SCHWAN, 2013).

Durante a fase aeróbica criada pela drenagem da polpa e o aumento da quantidade de ar, as bactérias ácido acéticas passam a dominar a fermentação. *Acetobacter pasteurianus* é a espécie que predomina a fermentação acética espontânea do cacau, que consiste na oxidação do etanol produzido pelas leveduras (LEFEBER et al., 2010). A distinção entre suas espécies, entretanto , é bastante dificultada pela homologia de suas sequências de rDNA; a presença de *A. tropicais*, *A. senegaensis* e *A. malorum* é constantemente relatada.

Nessa complexa comunidade microbiana, a identificação dos gêneros e espécies participantes é uma difícil tarefa pelas técnicas microbiológicas de cultura, já que muitas células perdem a viabilidade de cultivo, devido às condições de fermentação.

Comunidades microbianas variam em diversidade, abundância e funcionalidade, ao longo do processo fermentativo, e os membros não cultiváveis são essenciais para o entendimento de ecossistemas específicos e para o desenvolvimento de culturas *starters* (ILLEGHEMS et al., 2015).

O uso dos métodos de sequenciamento de nova geração (SNG) tem aumentado significativamente o conhecimento sobre a diversidade microbiana e sua função em ecossistemas microbianos complexos, tais como fermentações de alimentos que envolvem ecossistemas complexos (JUNG et al., 2011).

A metagenômica pode ser definida, resumidamente, como o estudo genético de uma amostra retirada de um ecossistema, já os dados das sequências de DNA constituem seu genoma microbiano coletivo, são denominados metagenoma (WOOLEY; GODZIK; FRIEDBERG, 2010). Dessa forma, por meio da metagenômica é possível o sequenciamento de DNA de toda a diversidade de uma comunidade, sem a necessidade do cultivo microbiano (LYU et al., 2013). Essa abordagem está sendo aplicada para desvendar a diversidade da microbiota envolvida no processo de fermentação das amêndoas de cacau (ILLEGHEMS et al., 2015), em que leveduras, bactérias láticas e acéticas são os principais agentes (SCHWAN; WHEALS, 2004).

1.2.3 Produção de chocolate

O chocolate possui duas grandes características que o distinguem: seu sabor e sua textura. Apesar de existir diversos tipos de chocolate, todos precisam ser livres de sabores indesejáveis e incorporar, pelo menos, alguns dos sabores que associam o consumidor ao produto. Um aspecto primário da textura é que o chocolate necessita ser sólido à temperatura ambiente (20 - 25°C) e derreter rapidamente na boca (37°C), se transformando em um líquido de consistência lisa para a língua. O processamento de chocolate é relacionado à obtenção desses dois critérios e, portanto, dedicado a desenvolver o sabor do produto e tratá-lo de forma que ele flua adequadamente e seja livre de materiais que promovam textura arenosa (BECKETT, 2009).

Existem diversos métodos de produção de chocolate, porém as principais etapas de processamento estão presentes em todos eles, mesmo que realizadas em equipamentos distintos.

Na produção tradicional, a primeira operação unitária que os grãos sofrem na fábrica é a torrefação. Ziegler (2009) ressalta que a torrefação do cacau ocorre em temperaturas entre 120°C e 140°C, remove a testa da semente e desenvolve o sabor característico de cacau, com a redução da umidade e acidez remanescentes da fermentação. Para a produção de chocolate e demais produtos crus, o cacau não é torrado.

Os grãos torrados, ou somente secos, são quebrados para facilitar o processo de moagem, passando a ser chamados de *nibs*. A massa de cacau é produzida com a moagem dos *nibs*, sendo liquida quando aquecida e sólida à temperatura ambiente. A moagem é extremamente fina, as partículas possuem cerca de 30 µm, para que o produto final não apresente textura arenosa e aparência opaca (KAMPHUIS, 2009).

O processamento do chocolate pode ser iniciado com a massa de cacau, aquecendo-a até sua liquefação, com o *liquor* de cacau, que já se encontra em estado líquido ou com o cacau em pó, misturando-o com a manteiga de cacau, previamente extraída. Os atributos sensoriais do chocolate são fortemente dependentes das proporções e distribuição espacial da massa de cacau, sólidos de leite e partículas de açúcar e das propriedades de cristalização da fase contínua, a manteiga de cacau (ROUSSEAU, 2006).

Muitos ingredientes em pó, como o açúcar, apresentam granulometria incompatível à requerida para a produção de chocolates, então são previamente misturados e refinados em moinhos de rolos. O tamanho das partículas pode variar de acordo com o produto final desejado, mas, geralmente, deve ser inferior a 40µm. A distribuição de tamanho das partículas governa as propriedades de escoamento do chocolate influenciando diretamente suas características sensoriais (BECKETT, 2009, SHAH; JONES; VASILJEVIC, 2010).

A conchagem é a etapa final, a última oportunidade de um fabricante obter o sabor requerido para o produto. Entretanto, ela não pode corrigir erros anteriores como, por exemplo, sabores desagradáveis de fumaça ou mofo provenientes de uma secagem ineficiente (BECKETT, 2009). É uma etapa necessária para remover os sabores indesejáveis provenientes da fermentação das sementes, principalmente o ácido acético remanescente. Melo (2008) diz que, durante a conchagem, são exercidas forças mecânicas cíclicas e de cisalhamento, a fim de separar os aglomerados formados na trituração, cobrir as partículas individuais com gordura e dispersar a fase de manteiga de cacau por todo o chocolate.

Na maioria das plantas de fabricação, a conchagem é precedida pelos moinhos de rolo, que transformam a massa de cacau em uma pasta quebradiça ou pó. A função da conchagem é dar continuidade ao processo fazendo com que a massa passe a um líquido capaz de fluir através do derretimento da gordura (BECKETT, 2009).

As tarefas físicas da conchagem são dispersar, desumidificar, remover componentes voláteis e homogeneizar, com o objetivo de melhorar a viscosidade, a fluidez e a textura e produzir um chocolate com boas características de fusão (MELO, 2008). Os ingredientes são misturados em aquecimento controlado, até que as partículas sólidas sejam recobertas pela manteiga de cacau. Se permanecerem superfícies não cobertas, haverá perda da sensação de derretimento, característica do chocolate ao entrar em contato com a saliva (BECKETT, 2009).

As temperaturas de conchagem variam entre 40 e 60°C. Os produtores de chocolate cru preconizam temperaturas mais baixas, para diminuir a interferência da temperatura na degradação de compostos bioativos.

Após a conchagem, a massa de cacau tem a formação de sabor finalizada e prossegue para as etapas de solidificação. A temperagem é uma dessas fases, sendo uma técnica de cristalização controlada, utilizada para induzir as formas polimórficas termodinamicamente mais estáveis da manteiga de cacau. Ocasiona um produto de qualidade com boa firmeza, contração, cor, brilho e vida de prateleira (AFOAKWA et al., 2009).

Durante o processamento, a cristalização da manteiga de cacau possui um importante papel na obtenção de um produto de alta qualidade. As características estruturais ideais são ditadas pela rede cristalina formada pela cristalização de seus componentes lipídicos, tornando a temperagem uma operação unitária crucial na fabricação de chocolates. O chocolate mal cristalizado provoca a formação do *fat bloom*, um filme superficial acinzentado (AFOAKWA et al. 2009; FERNANDES; MÜLLER; SANDOVAL, 2013).

As transições de fases no sistema polimórfico ocorrem das formas menos estáveis para as mais estáveis e são geralmente irreversíveis e tempo-temperatura dependentes. Acredita-se que essas transformações ocorrem diretamente na manteiga de cacau completamente derretida, com exceção das duas fases β (β_V e β_{VI}) (FERNANDES; MÜLLER; SANDOVAL, 2013).

A temperagem é um processo no qual o chocolate é termicamente tratado para produzir uma pequena fração de cristais de gordura altamente estáveis do tipo e tamanhos corretos (β_V) e dispersos homogeneamente na massa. Esses cristais crescem formando uma rede microcristalina homogênea, durante os estágios sequentes de moldagem e resfriamento (WINDHAB, 2009).

Existem duas principais formas de moldagem. A primeira é enformar o chocolate temperado utilizando um molde. Essa pode ser uma simples operação, no caso de formar tabletes sólidos, ou pode incluir a formação de cascas de chocolate que receberão um recheio. A cobertura é outro método e utiliza um recheio pré-formado, sobre o qual o chocolate temperado será vertido (GRAY, 2009).

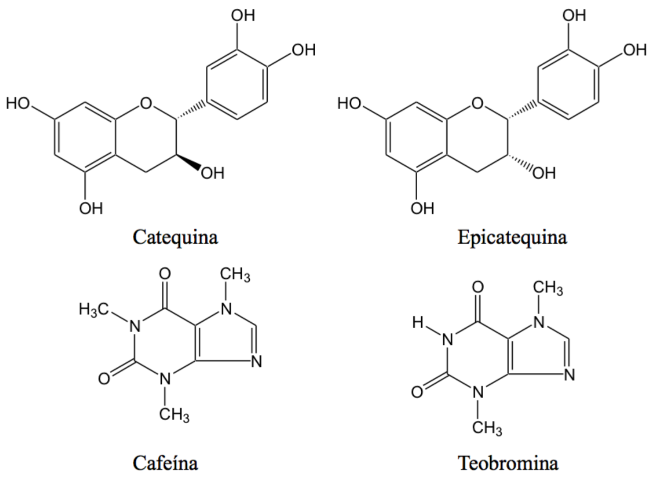
No caso da moldagem com formas, após o chocolate temperado ser despejado sobre elas é iniciada a vibração mecânica, que acomoda o chocolate e libera as bolhas de ar. Este tipo de moldagem resulta em um brilho mais aparente no produto final. Os moldes com o chocolate temperado são resfriados, provocando a remoção do calor especifico latente e, como consequência, a contração da massa, dando-lhe consistência e facilitando a retirada do chocolate dos moldes (OETTERER, 2006; MELO, 2008).

A necessidade de cristalizar a gordura em pequenos cristais na forma β_V limita a taxa de resfriamento que pode ser aplicada. O resfriamento rápido ou o emprego de temperaturas muito baixas resultam em formas polimórficas menos estáveis, como a β_{IV} (GRAY, 2009). Se a temperagem e o resfriamento foram otimizados, então a retirada da forma torna-se menos crítica no processo, resultando em produtos de boa qualidade, que saem totalmente da forma, são estáveis e atrativos (MELO, 2008).

1.2.4 Compostos bioativos do cacau e derivados

Os compostos bioativos são substâncias encontradas em alimentos e classificadas como ingredientes com atividade biológica. No cacau, os bioativos de maior concentração são os compostos fenólicos e as metilxantinas, sendo consideradas as substâncias de maior interesse científico e industrial para seus derivados. Os compostos fenólicos e metilxantinas são produtos naturais, conhecidos como metabólitos secundários produzidos pelos vegetais, apresentando funções ecológicas importantes, como: proteção contra herbívoros e patógenos, ação alelopática, além de agir como atrativos para animais polinizadores (TAIZ; ZEIGER, 2004). Na figura 1.1 são apresentados os compostos fenólicos e metilxantinas presentes em maior concentração no cacau.

Fig.1.1 Fórmula estrutural dos principais fenólicos e metilxantinas presentes no cacau e derivados.



Catequina

Epicatequina

Cafeína

Teobromina

O grupamento dos flavonoides abrange os flavonóis, antocianinas, flavonas e flavanonas. Entre estes, os flavanóis são os mais abundantes, sendo a (+)-catequina e a (–)-epicatequina os principais representantes. A (–)-epicatequina tem sido reportada como o principal flavanol monomérico do cacau (WOLLGAST; ANKLAM, 2000; EFRAIM et al., 2006).

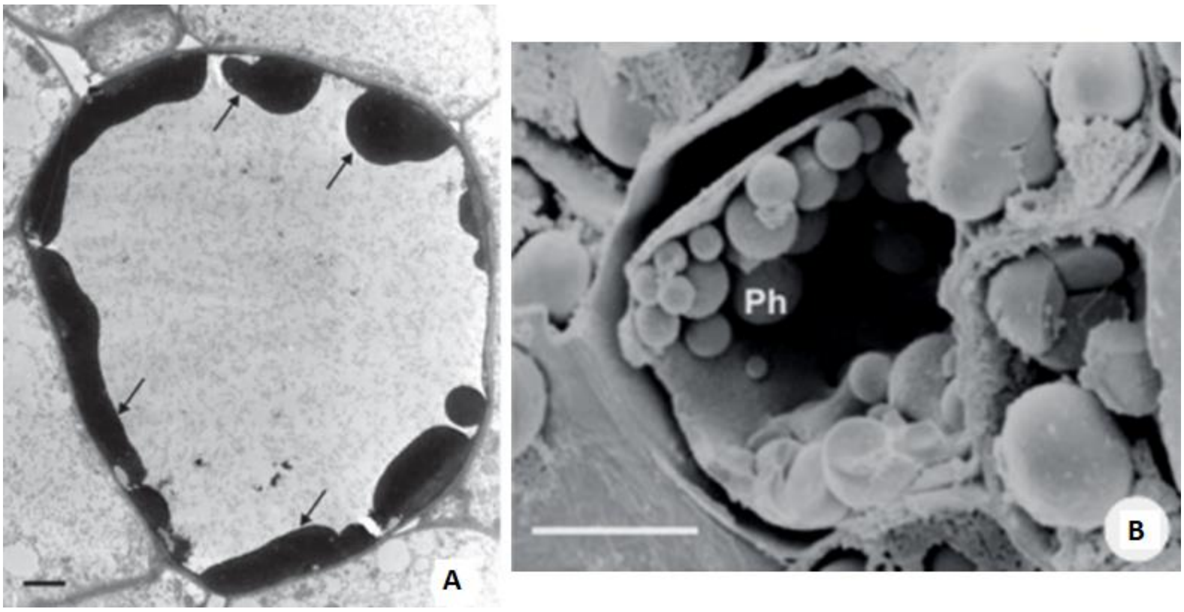
A catequina, a epicatequina e as procianidinas não são compostos exclusivos do cacau, aparecem também em outros produtos como cereja, pêssego, maçã, chá verde e uvas, mas é no cacau onde estão as maiores concentrações (FERNÁNDEZ-MURGA, 2011).

Os flavonoides podem agir como antibióticos, pesticidas naturais, atraentes para polinizadores, agentes protetores contra a luz ultravioleta e constituem materiais para tornar a parede celular impermeável à água e como material estrutural para dar estabilidade à planta. Em alimentos, os fenólicos podem contribuir para o amargor, adstringência, pigmentação, sabor, odor e estabilidade oxidativa dos produtos (NACZK; SHAHIDI, 2004).

Os benefícios nutricionais do cacau vêm sendo estudados e a importância do seu consumo enfatizada, uma vez que as descobertas científicas têm sido positivas (THAMKE; DURRSCHMID; ROHM, 2009). Pesquisas têm mostrado que, no cacau, encontra-se uma grande quantidade de compostos fenólicos em suas folhas e, principalmente, em suas sementes. Nestas, os compostos fenólicos são armazenados nas células de pigmentos (Fig. 1.2). Dependendo da quantidade desses compostos, a coloração das células varia de branca a púrpura (OSMAN et al., 2004).

Além dos compostos fenólicos, tem-se a presença das metilxantinas que são responsáveis por alterações no organismo humano, agindo sobre os sistemas nervoso central, cardiovascular, renal e digestivo. Os efeitos são qualitativamente semelhantes, mas quantitativamente diferentes, em função disso, são empregadas com diferentes finalidades terapêuticas (BRUNETON, 1991).

Fig.1.2 (A) Microscopia de célula de *T. cacao* com vesículas de polifenóis aderidas à membrana plasmática. Microscopia eletrônica de transmissão de célula com armazenamento de compostos fenólicos. (MARTINI et al., 2008).



No cacau e no chocolate, a metilxantina predominantemente encontrada é a teobromina, seguida da cafeína, em quantidades menores (ROZIN et al., 1991). Os efeitos fisiológicos na saúde humana mais comuns da cafeína incluem estimulação do sistema nervoso central, sendo utilizada no tratamento de dores de cabeça comuns, também dos músculos cardíacos, do sistema respiratório e da secreção de acido gástrico. Também é considerada como um diurético fraco e relaxante muscular. A teobromina (3,7-dimetilxantina), encontrada, sobretudo, em produtos de cacau, tem ação diurética, estimulante, com menor efeito sobre o sistema nervoso central (BRUINSMA; TAREN, 1999; OSMAN et al., 2004).

A teobromina, diferentemente da cafeína, estimula pouco o sistema nervoso central humano, além de suprimir a atividade do nervo sensorial e interferir na tosse, tem sido proposta para ajudar no tratamento dietético da aterosclerose, e pode contribuir positivamente para o controle da pressão arterial, a partir do aumento no consumo do cacau e do chocolate amargo (WEISBURGER; CHUNG, 2002).

O consumo de chocolate tem efeito positivo em indivíduos em estado de ansiedade, associado à redução dos níveis de normetanefrina, adrenalina, corticoesterona, noradrenalina, progesterona, leucina, cortisol e asparagina (MATIN et al, 2009).

Estudos relacionados a algumas bebidas largamente consumidas, como café e chá, indicam a ação da cafeína sobre a musculatura estriada e como estimulante do sistema nervoso central, enquanto que a teobromina aumenta o fluxo sanguíneo renal e a filtração glomerular (SIMÕES et al., 2004).

Martin et al. (2009) comprovaram os efeitos *in vivo* do consumo diário de chocolate amargo ao constatarem a alteração do metabolismo, redução dos hormônios associados ao stress e normalização dos sistemas de oxidação metabólica.

1.3 OBJETIVO GERAL

Avaliar o cacau cru produzido na região IG Sul da Bahia e seus derivados, quanto ao teor de compostos bioativos, atividade antioxidante e microbiota fermentadora.

1.4 OBJETIVOS ESPECÍFICOS

- Caracterizar os compostos fenólicos e alcalóides do chocolate cru.
- Quantificar os compostos bioativos presentes na amêndoa e na testa do cacau cru, verificando as potencialidades de uso alimentício e industrial.
- Verificar a contribuição dos compostos solúveis e insolúveis no perfil antioxidante dos produtos analisados.
- Identificar a microbiota fermentadora autóctone, empregando metagenômica.

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CAPÍTULO 2

SOLUBLE AND INSOLUBLE-BOUND FRACTIONS OF PHENOLICS AND ALKALOIDS AND ANTIOXIDANT ACTIVITIES IN RAW AND TRADITIONAL CHOCOLATE: A COMPARATIVE STUDY

(Artigo publicado na revista journal of functional foods)

Soluble and insoluble-bound fractions of phenolics and alkaloids and antioxidant activities in raw and traditional chocolate: a comparative study

Abstract

Raw chocolate, produced with unroasted cocoa beans, is gaining the attention of the industry and consumers. In the present study, alkaloids and phenolics present in the soluble and insoluble-bound forms were identified and quantified by ultra-high performance liquid chromatography. The antioxidant activity was investigated with four methods (ABTS, DPPH, ORAC, and FRAP). Extraction of insoluble-bound components was critical for accurate quantification of theophylline, protocatechuic acid, catechin, as well as epigallocatechin and contributed to better understanding of the antioxidant activity of the test samples. Raw formulations showed higher quantities of many compounds present in the soluble form in comparison to the concentration found in traditional formulations. Roasting promoted the release of protocatechuic acid, catechin, epigallocatechin and theophylline from the cocoa cell walls**.** This is the first screening of the soluble and insoluble-bound antioxidants in both traditional and raw chocolate, therefore impacting the understanding of the chemistry of chocolate.

Keywords: Unroasted cocoa; Processing effects; Bioactive compounds; UPLC- DAD; Methylxanthines; Phenolic Profile.

2.1 INTRODUCTION

Chocolate has unique sensory characteristics which stems from the presence of several compounds that act as flavor precursors. However, these natural compounds may also exhibit important biological activities. Of particular interest are the groups of alkaloids, responsible for the stimulating effect of the central nervous system, which increases cognitive function and alertness. These natural compounds may also exert benefits to gastrointestinal, cardiovascular, renal and respiratory systems (DE SENA; DE ASSIS; BRANCO, 2011; MARTÍNEZ-LÓPEZ et al., 2014; ASHIHARA et al., 2015; ALAÑÓN et al., 2016). Phenolic compounds, another group of bioactives present in chocolate, are also related to many physiological effects. The antioxidant properties of food phenolics are well substantiated. Likewise, their rule in potentially reducing the risk of development of several chronic ailments such as cardiovascular, neurological and inflammatory diseases, some types of cancers and type 2 diabetes has recently been reported (CARRILLO; LONDOÑO-LONDOÑO; GIL, 2014; PATRAS et al., 2014; BORDIGA et al., 2015; DE CAMARGO et al., 2015; ZHANG; TSAO, 2016; AMAROWICZ; PEGG, 2017; SOUZA et al., 2017; GIANFREDI et al., 2018).

Roasting, a common stage in cocoa postharvest handling and processing, may affect the concentration of compounds of biological interest (BORDIGA et al., 2015; DI MATTIA et al., 2017). The effect of roasting in other commodities such as coffee beans and their industrial products indicates that high temperatures may be detrimental to some bioactive compounds (VIGNOLI et al., 2014). These findings have boosted the number of investigations on the subject and as a consequence the demand for green coffee products by consumers has increased. The same behavior has been observed in the chocolate market as recently the presence of raw chocolate, the product elaborated with unroasted beans, has been increasing (ŻYŻELEWICZ et al., 2018). The product has a more intense and acidic flavor and is currently produced in several countries. Producers of raw chocolate have been using high quality feedstocks such as fine cocoa from organic crops and/or with geographical indication. However, the potential benefits of higher concentrations of bioactive compounds in raw chocolate has not yet been entirely clarified.

Soluble free and conjugated phenolic compounds are stored in the vacuoles of plant cells while insoluble-bound compounds are linked to the cell walls of plant materials (SHAHIDI; YEO, 2016; DE CAMARGO et al., 2017). However, their contributions and hence their biological activities may differ among different fractions. Soluble phenolics can be absorbed in the small intestine and may enter the bloodstream thus acting in the cells and tissues. In contrast, insoluble-bound compounds reach the large intestine, where they may be hydrolyzed by enzymes produced by the intestinal microbiota. The release of phenolics in the colon promotes beneficial effects, such as lowering the pH, development of fermentative microflora and preventing the growth of pathogenic and carcinogenic bacteria (RODRÍGUEZ-ROQUE et al. 2014; SHAHIDI; YEO, 2016). Insoluble-bound phenolics have been identified in several feedstocks as grapes (TOSCANO et al., 2017), winery by-products (MELO et al., 2015; DE OLIVEIRA et al., 2017), peanuts and its by-products (DE CAMARGO et al., 2015; DE CAMARGO et al., 2017), barley and malt (DVOŘÁKOVÁ et al., 2008), millet grain (CHANDRASEKARA; SHAHIDI, 2012) and berry seed meals (AYOUB; DE CAMARGO; SHAHIDI, 2016). Likewise, the presence of other groups of bioactives such methylxanthines and other alkaloids in the insoluble-bound fraction may also be contemplated. Therefore, such investigation is deemed necessary. Furthermore, studies on the quantification and bioactivity of soluble phenolics are easily found for the most diverse food products. In contrast, analysis of insoluble bound compounds is less or sometimes rarely explored or in some cases is even inexistent. Therefore, quantification of compounds and the contribution of both fractions to the antioxidant activity were investigated in this work. Finally, such studies would also contribute to a better understanding of the effects in producing traditional versus raw chocolate by screening relevant chemical biomarkers.

2.2 MATERIAL AND METHODS

2.2.1. Chemicals and reagents

Standards of alkaloids (theobromine, caffeine, paraxanthine, theophylline, nicotinic acid and trigonelline) and phenolic compounds (catechin, epicatechin, epigallocatechin, rutin, myricetin, quercetin, kaempferol, trigonelline, gallic acid, caffeic acid, protocatechuic acid, ascorbic acid, ferulic acid, sinapic acid, *p*-coumaric acid and chlorogenic acid) were purchased from Sigma Aldrich (St. Louis, USA). Chromatographic solvents, methanol and formic acid, were HPLC-grade (LiChrosolv®, Merck, Darmstadt, Germany). The reagents and solvents (acetone, acetic acid, calcium carbonate, ethanol, ethyl acetate, ethylic ether, ferric chloride, ferric sulfate, hexane, hydrochloric acid, methanol, potassium persulfate, potassium phosphate, and sodium hydroxide) utilized in extractions and spectrophotometric methods: were of analytical grade (Anidrol, Diadema, Brazil). The reagents and standards (2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{TM}); 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein, and Folin-

Ciocalteu’s reagent) utilized in spectrophotometric methods were purchased from Sigma Aldrich, St. Louis, USA.

2.2.2. Chocolate production

As in any other crop, the biochemical composition of seeds is highly dependent on the subspecies, geographical origin and climatic conditions of cultivation, thus the same cocoa was used during the elaboration of chocolates and the same processing conditions were applied, except for cocoa roasting. Traditional chocolates were evaluated here to serve as control samples. Forastero organic cocoa was cultivated in traditional Brazilian agroforestry system in the city of Itacaré, Bahia State, Brazil. The fruits were harvested at the same day and remained at rest for 4 days. The pods were broken and the seeds with the pulp were fermented in wood boxes for five days. The seeds were then washed with water and dried in a semi-opened greenhouse. One portion from the dried seeds was reserved to produce raw chocolates and the other was roasted under mild temperature (up to 100°C) for 20 min. Four chocolate formulations were produced: traditional 70% and raw 70% (with addition of 28% of sugar and 2% of cocoa butter) and traditional 85% and raw 85% (with addition of 15% of sugar). The sugar added was granulated organic demerara (Native, Sertãozinho, Brazil) and the organic raw cocoa butter (IBC Cacau, Ilhéus, Brazil) was produced from beans from the same farm. The ingredients were previously grounded in a roller mill and conched in a melangéur (ECGC Cocoatown, Roswell, USA) for six hours, following the procedure conducted by the farmers for commercial chocolate, with maximum temperature in all processes controlled at 45° C. The chocolate was placed in acetate molds and refrigerated until its solidification, packed in aluminum foil and stored at room temperature until the moment of analysis, which took months.

2.2.3. Extraction process

The extracts containing soluble and insoluble-bound compounds were obtained according to the method described by de Camargo, Regitano-d’Arce, Gallo and Shahidi (2015). The samples (40 g) were defatted with hexane (solid/solvent, 1:5, w/v) in a blender (Avance, Philips Walita, Varginha, Brazil) and the mixture was filtered (Whatman 40, low ash, 8 µm pore size) using a vacuum pump. The procedure was repeated three times. The solids were dried at 40°C for 4 hours in an air circulating oven (MCientifica 420, Mogi das Cruzes, Brazil) and stored at -20°C until subsequent extractions. Soluble phenolics were extracted with 70% acetone (solid/solvent, 1:40, w/v) in a water bath shaker (NT-295, Nova Técnica, Piracicaba, Brazil) at 30°C for 20 min. After centrifugation at 4000 x *g* (5084R, Eppendorf AG, Hamburg, Germany), the supernatant was collected. The extraction was repeated two more times and the supernatant was combined. Acetone was evaporated under vacuum in a rotary evaporator (Model 801 Fisatom, São Paulo, Brazil) at 40°C and then lyophilized. Yields of extraction ranged from 84 mg to 153 mg of soluble compounds per g of chocolate. Extracted compounds were reconstituted in HPLC-grade methanol and stored at -20 °C. The residue remaining after the extraction of soluble compounds–was hydrolyzed with 4M NaOH (1:1, v/v) at 40°C for 4 hours following acidification to pH 2 with 6 M HCl. Insoluble-bound compounds released were extracted with diethyl ether and ethyl acetate (1:1, v/v). The process was repeated five times and the solvent was evaporated in a rotary evaporator at 40°C.

Yields of extraction ranged from 39 mg to 66 mg of insoluble compounds per g of chocolate. Dried extracts were resuspended in HPLC-grade methanol.

2.2.4. Determination of methylxanthines, alkaloids, and phenolics compounds by UHPLC

Methanolic extracts were injected in a Waters HSS C18 column, 1.8 μm, 2.1 mm X 100 mm (Waters, Dublin, Ireland) attached in an UPLC® Acquity (Waters, Milford, MA, USA) coupled to a thermostatic autosampler (5°C), binary solvent manager, column oven (40°C) and diode-array detector (DAD) set at 270 nm was used. The mobile phase consisted of solvent A: water and solvent B: methanol and both were acidified with formic acid (0.1% v/v). The flow rate was 0.4 $mL.min^{-1}$ following the gradient: 0 min, 5% B and 95% A; 10 min, 95% B and 5% A; 10.1 min, 5% B and 95% A.

Total run time was 13 min. Limit of detection (LOD) was defined from visual inspection in 0,03 mg/L. Quantification was carried out with calibration curves from external standards with linear curves from 0,1 mg/L to 100 mg/L, defining the limit of quantification (LOQ) as 0,1 mg/L. Representative UPLC-DAD chromatograms for the soluble and insoluble-bound fractions are presented in Fig.2.1.

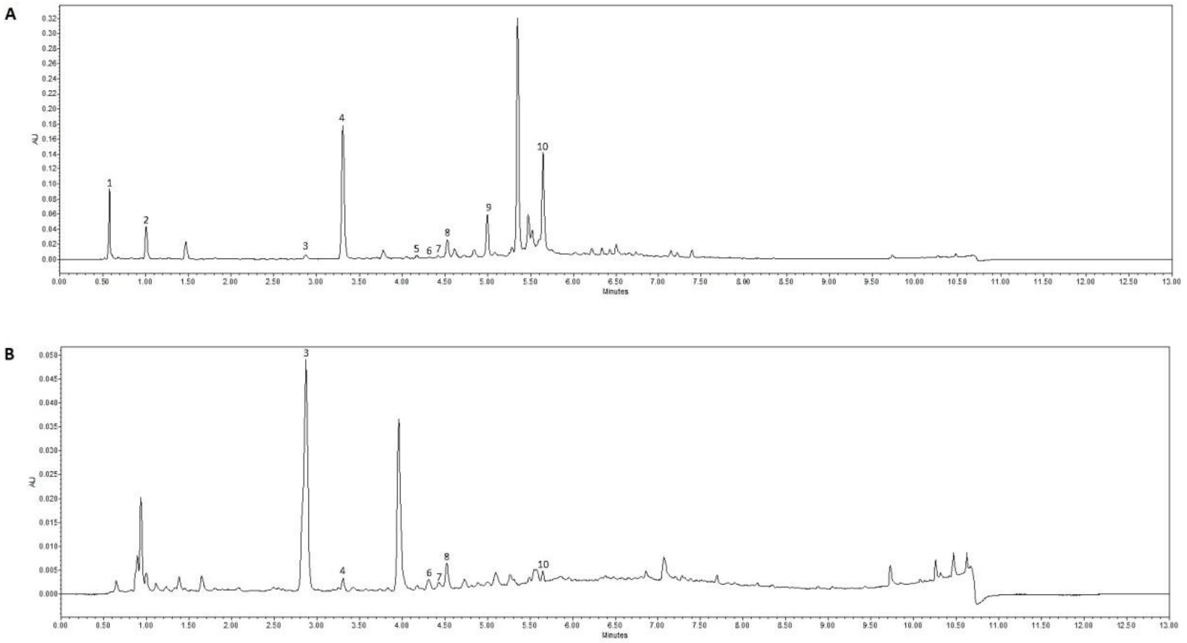

Fig. 2.1. Representative UPLC-DAD chromatograms from Raw 85%. (A) Soluble compounds (B) Insoluble-bound compounds. Identified peaks: (1) Trigonelline RT=0,520 (2) Nicotinic acid RT=0,835 (3) Protocatechuic acid RT=2,871 (4) Theobromine RT=3,304 (5) Paraxanthine RT=4,167 (6) Theophylline RT= 4,317 (7) Epigallocatechin RT = 4,418 (8) Catechin RT = 4,525 (9) Caffeine RT = 5,346 (10) Epicatechin RT = 5,644.

2.2.5. Total phenolic content (TPC)

Total phenolic contents were determined according to the procedure described by de Camargo et al. (2015) with minor modifications. Phenolic extracts (0.5 mL) were mixed with deionized water (4 mL) and Folin-Ciocalteu's reagent (0.5 mL). After three min, a saturated sodium carbonate solution (0.5 mL) was added and the flasks were kept in the dark for 2 hours. The absorbance was read at 760 nm in a UV-visible spectrophotometer (Libra S22, Biochrom, Cambridge, UK). The results expressed as milligram of gallic acid equivalents per gram of chocolate.

2.2.6. Ferric reducing antioxidant potential (FRAP)

The ferric reducing antioxidant potential was carried out according to Sánchez-González, Jiménez-Escrig and Saura-Calixto (2005) with minor modifications. The stock solution consisted of 0.3 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ solution. Acetate buffer (25 mL) and

TPTZ (2.5 mL) were mixed, and 2.5 mL $FeCl_3$ were added. Methanolic phenolic extracts (10 µL) were added to 900 µL of the FRAP solution and kept for 30 min in the dark. The absorbance was measured at 595 nm using a spectrophotometer (Model Libra S22, Biochrom, Cambridge, UK). The results were expressed as µmol Fe(II) per gram of chocolate.

2.2.7. DPPH radical scavenging assay

The scavenging capacity was using the DPPH radical-scavenging method described by Brand-Williams, Cuvelier and Berset (1995), with minor adaptations. Briefly, the diluted methanolic extracts (500 µL) were added to ethanol (3 mL) and the DPPH solution (0.5 mM - 300 µL). After 45 minutes in the dark, the absorbance was read at 515 nm using UV-visible spectrophotometer (Libra S22, Biochrom, Cambridge, UK). The calibration curve was made with methanolic solutions of Trolox at different concentrations (200 to 2000 µM). The DPPH scavenging capacity was expressed as µM Trolox equivalent per gram chocolate.

2.2.8. ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was conducted according to Re et al. (1999). The radical $ABTS^{\bullet+}$ was previously prepared by mixing the ABTS stock solution 7 mM (5 mL) with potassium persulfate 150 mM (88 µL). The solution was left in the dark for 16 hours. The absorbance at 734 nm was then adjusted to 0.700 nm ± 0.050 with ethanol by using a Libra S22 spectrophotometer (Biochrom, Cambridge, UK). The reaction was conducted with methanolic extracts (30 µL) and the radical $ABTS^{\bullet+}$ solution (3 mL). The standard curve was calculated with Trolox

(100 to 2000 μM) and the results expressed in μmol Trolox equivalent per gram chocolate.

2.2.9. Oxygen radical absorbance capacity (ORAC*)*

The ORAC assay was performed as described for Melo et al. (2015) in a microplate reader (Molecular Devices Corp., Sunnyvale, CA). Briefly, 30 μL of the standard, blank or sample were mixed directly in the microplate with 60 μL of fluorescein solution 508 mM. Subsequently, 110 μL of AAPH solution was injected in each well to initiate the reaction, following incubation at 37 °C for 15 min. The fluorescence was determined every min for 2 hours with an excitation and emission wavelengths of 485 and 528 nm, respectively. Different concentrations of Trolox were used to prepare the standard curve (12.5 to 400 μM). The results were expressed as μmol of Trolox equivalents per gram of chocolate.

2.2.10. Statistical analysis

Spectrophotometric analysis were carried out in triplicates ($n = 3$) and UPLC in duplicates ($n = 2$). One-way analysis of variance (ANOVA) and least significant difference (LSD) *post hoc* Tukey’s test were used to compare the results ($P < 0.05$). Pearson correlation coefficients were also calculated. All analysis were conducted using the software Statistica 13.3 version 64 (TIBCO Software Inc., Palo Alto, USA).

2.3. RESULTS AND DISCUSSION

2.3.1. Total phenolic content (TPC)

The TPC of raw and traditional chocolates is presented in Fig. 2.2. Considering both soluble and insoluble-bound fractions the highest TPC value was found in the raw 85% sample (53.9 mg GAE g^{-1}) whereas the lowest one was found in traditional 70% sample (15.8 mg GAE g^{-1}). Our results (Fig. 2.2) also show that raw chocolate contain more polyphenols than the traditional version. As expected, the TPC increases with the cocoa content. Di Mattia et al. (2014) found the range between 39.1-39.9 mg GAE g^{-1} in conched chocolate mass.

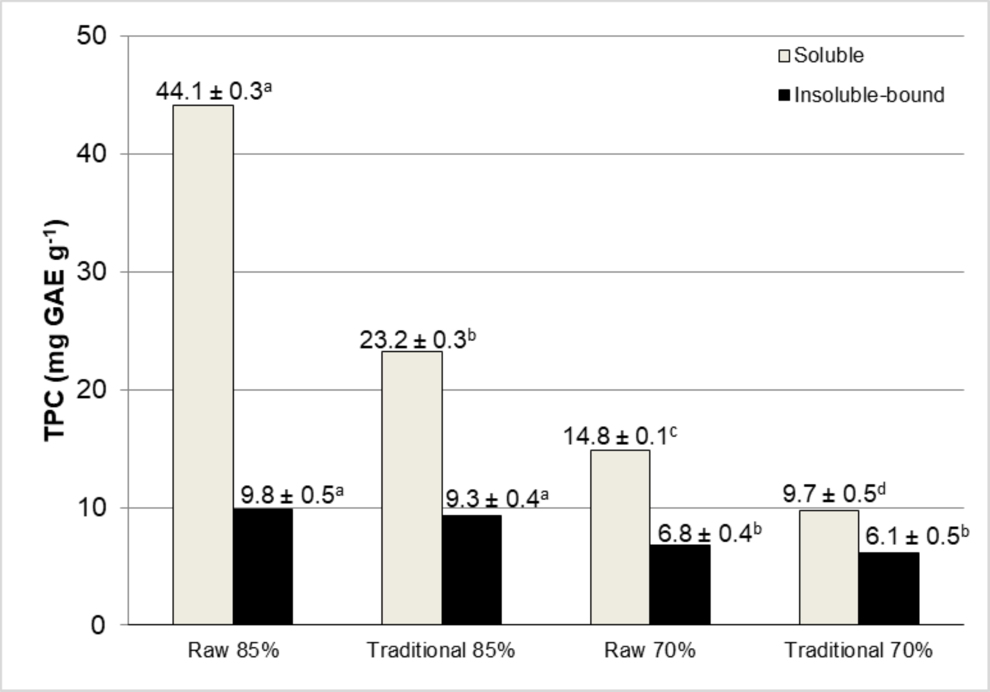

Fig. 2.2. TPC in raw and traditional chocolates. Values are the mean ± S.D. (N=3). Means with different letters within each fraction show difference between samples in Tukey test ($p \leq 0.05$).

The TPC ranged from 7.09 to 31.3 mg GAE g^{-1} (BRCANOVIĆ et al., 2013) and from 20.0 to 36.62 mg GAE g^{-1} (VERTUANI et al., 2014) in studies conducted with dark chocolates (70% - 100% cocoa solid content). In a study of discrimination of cocoa bean origin by chocolate polyphenols, Cambrai et al. (2017) found the highest levels of compounds in Brazilian samples produced with forastero type, in comparison with chocolates obtained from cocoa originated from 12 countries. These data show that the TPC values from the present study are in good agreement with the literature. The high levels of phenolics found may be interesting for the determination of GI in this area, as Bahia State is in process of obtaining the GI certification for cocoa products. Therefore, the present results are also important for both local and global chocolate producers as well as for their recognition in national and international markets.

The separated fractions evidenced that soluble phenolics were more susceptible to thermal degradation, while insoluble-bound phenolics remained quite stable. The contribution of insoluble-bound phenolics was significant, especially for the traditional 70% chocolate (up to 38.6% of TPC), thus demonstrating that phenolics linked to the cocoa cell wall constituents should not be underestimated. To the best of our knowledge, this is the first report on the contribution of insoluble-bound phenolics in chocolate; hence, our data fills this timely relevant and apparent gap.

2.3.2. Phenolic compounds

The contents of soluble and insoluble-bound phenolic compounds in raw and traditional chocolates are shown in Table 2.1. The soluble fraction was the main contributor in the phenolic profiling, except for the concentration of protocatechuic acid. In general, roasting promoted the reduction of all soluble compounds. This is in good agreement with the literature (BORDIGA et al., 2015; DI MATTIA et al., 2017) as roasting has been found to play an important impact in degrading polyphenols.

Catechin and epicatechin are monomeric flavonoids able to neutralize free radicals. Their antioxidant activity has been reported to be lower than that of oligomeric proanthocyanidins. However, their lower molecular weight makes them more capable of cross the intestinal barrier thus becoming bioavailable to act in organs and plasma, while condensed forms depend of prior metabolization in the gut and may have a higher local activity (JIMENEZ-RAMSEY et al., 1994; CHANDRASEKARA; SHAHIDI, 2012).

Table 2.1 The contents of soluble and insoluble-bound compounds (mg g^{-1}) of raw and traditional chocolates with different cocoa solids contents.

	Raw 85%	**Traditional 85%**	**Raw 70%**	**Traditional 70%**
Epicatechin				
Soluble	36.812 ± 0.002^{aA}	29.624 ± 0.325^{bA}	16.801 ± 0.169^{cA}	4.395 ± 0.040^{dA}
Insoluble-bound	0.287 ± 0.008^{aB}	0.271 ± 0.002^{aB}	0.083 ± 0.003^{bB}	0.059 ± 0.003^{bB}
Catechin				
Soluble	16.413 ± 0.114^{aA}	15.909 ± 0.089^{bA}	4.797 ± 0.029^{cA}	4.175 ± 0.018^{dA}
Insoluble-bound	2.193 ± 0.015^{bB}	2.7642 ± 0.029^{aB}	0.394 ± 0.013^{dB}	0.746 ± 24.85^{cB}
Epigallocatechin				
Soluble	0.184 ± 0.001^{aA}	0.156 ± 0.001^{bA}	0.065 ± 0.001^{cA}	0.065 ± 0.001^{cA}
Insoluble-bound	0.051 ± 0.001^{bB}	0.066 ± 0.002^{aB}	0.018 ± 0.002^{cB}	0.025 ± 0.005^{cB}
Protocatechuic acid				
Soluble	0.778 ± 0.022^{aB}	0.704 ± 0.001^{bB}	0.268 ± 0.009^{cB}	0.025 ± 0.001^{dB}
Insoluble-bound	4.465 ± 0.010^{aA}	1.874 ± 0.094^{bA}	1.073 ± 0.011^{cA}	0.619 ± 0.020^{dA}

Data are presented as mean ± standard deviation (n = 2). Values within the same row followed by the same lower case letters are not significantly different according to Tukey's Test ($p < 0.05$). Values within the same column, referring soluble and insoluble-bound fractions of the same compound, followed by the same capital letters are not significantly different according to test t ($p< 0.05$). Abbreviations: nd, not detected.

In the soluble fraction, epicatechin was the major flavonoid detected in the samples (4.395 mg g^{-1} to 36.812 mg g^{-1}) whereas the concentration of catechin ranged from 4.175 mg g^{-1} to 16.413 mg g^{-1}. Epigallocatechin was found in minor quantities but increased with cocoa content (from 0.065 mg g^{-1} to 0.184 mg g^{-1}). The presence of a pyrogallol group in catechin, forming epigallocatechin, enhances the antioxidant capacity (PIETTA, 2000), making their presence interesting even in minor concentrations. Tokusoglu and Ünal (2002) evaluated commercial chocolate formulations from Turkey with diverse cocoa solids contents. According to these authors, the concentration of epicatechin ranged from 0.11 mg g^{-1} to 8.39 mg g^{-1}, catechin ranged from 0.11 mg g^{-1} to 0.72 mg g^{-1} and epigallocatechin ranged from 0.05 mg g^{-1} to 0.39 mg g^{-1}.

Even though the cocoa origin of the analyzed chocolates was not considered, their study is useful to highlight the high levels found in the present work. Catechin, epicatechin and epigallocatechin were also found in insoluble-bound extracts (Table 2.1). While the concentration of epicatechin was different only between chocolates with distinct cocoa content, which increased with the level of cocoa in the formulation, the concentration of epigallocatechin was different also between processes (only for 85% formulations). Furthermore, the content of catechin increased upon roasting. Payne et al. (2010) explained that even in mild temperatures, above 70°C, roasting increases catechin level due to the epimerization of epicatechin in the heat treatment. Proanthocyanidins (condensed tannins) are not subject of this study but may help to explain this behavior. Condensed tannins consist mainly of catechin and epicatechin and high temperatures upon roasting may promote their depolymerization which may enhance the levels catechin. Quiroz-Reyes and Fogliano (2018) also attributed the increase in the catechin concentration to the degradation of procyanidins into their free flavan-3-ol monomers units, like catechin and epicatechin due to the high temperature of roasting. This observation suggests that chocolate can present high levels of insoluble-bound proanthocyanidins, a fact that may be addressed in future studies.

Protocatechuic acid, a hydroxybenzoic acid derivative, has been reported to have a strong antioxidant activity. It may also have a role reducing the risks of development of ulcers, atherosclerosis, and certain types of cancer. Such properties have been attributed to its ability in inhibiting the generation of free radicals and/or by directly blocking binding sites of carcinogens with DNA molecules (KAKKAR; BAIS, 2014). Its occurrence in chocolates is not often reported as a major compound, but an important amount was identified in this work (Table 2.1). It is also important to mention that about 85% of protocatechuic acid was present in the fraction containing phenolics released from their insoluble-bound form. According to Brcanović et al. (2013), the concentration of protocatechuic acid was in the range of 61 µg g^{-1} to 75 µg g^{-1} in dark chocolates. These values are considerably lower than found in the present study (adding up soluble and insoluble-bound amounts: 644 µg g^{-1} to 5232 µg g^{-1}). The predominance of protocatechuic acid in the insoluble-bound fraction makes the extraction method a crucial step to the global understanding of phenolic compounds in chocolates. The increase of 15% of cocoa solids content (from 70 to 85%) showed a great impact in soluble epicatechin, up to 2.2- and 7-fold, in raw and traditional formulations, respectively. With protocatechuic acid levels, it reflects in up to 2.9- and 28-fold in the soluble fraction and up to 4.1- and 3-fold in insoluble-bound fraction, in raw and traditional formulations, respectively.

Changes in the content of polyphenols during the production of chocolate are results of high temperature and the presence of oxygen, which accelerate oxidative processes (BORDIGA et al., 2015). Therefore, raw chocolate can be considered as a better dietary source of phenolic compounds in comparison to traditional formulations.

2.3.3. Alkaloids

The contents of soluble and insoluble-bound theobromine, caffeine, paraxanthine, theophylline, nicotinic acid and trigonelline in traditional and raw chocolates are shown in Table 2.2. Theobromine was in the range of 17.861 mg g^{-1} to 40.103 mg g^{-1} in the fraction containing soluble compounds. These values are extremely high comparing to quantifications from the literature, that report a range from 0.85 mg g^{-1} to 1.05 mg g^{-1} (RAMLI et al., 2006); 6.14 mg g^{-1} to 8.26 mg g^{-1} (BORDIGA et al., 2015), and 0.50 mg g^{-1} to 13.7 mg g^{-1} (ALAÑÓN et al., 2016), in commercial dark chocolates samples. This great variability may be explained by different cocoa growing conditions, mainly because of the altitude among other reasons (CARRILLO; LONDOÑO-LONDOÑO; GIL, 2014). Therefore, the cocoa used in this work may be considered a natural rich source of these and other bioactive compounds.

Table 2.2 The contents of soluble and insoluble-bound xanthines (mg g^{-1}) of raw and traditional chocolates with different cocoa solids contents.

	Raw 85%	**Traditional 85%**	**Raw 70%**	**Traditional 70%**
Theobromine				
Soluble	40.103 ± 0.088^{aA}	30.934 ± 0.047^{bA}	18.673 ± 0.070^{cA}	17.861 ± 0.080^{dA}
Insoluble-bound	0.234 ± 0.001^{aB}	0.234 ± 0.005^{aB}	0.205 ± 0.005^{aB}	0.217 ± 0.041^{aB}
Caffeine				
Soluble	28.584 ± 0.043^{a}	23.362 ± 0.074^{b}	10.312 ± 0.051^{c}	9.598 ± 0.027^{d}
Insoluble-bound	nd	nd	nd	nd
Nicotinic acid				
Soluble	0.402 ± 0.001^{a}	0.107 ± 0.001^{c}	0.236 ± 0.007^{b}	0.023 ± 0.001^{d}
Insoluble-bound	nd	nd	nd	nd
Paraxanthine				
Soluble	0.378 ± 0.001^{a}	0.310 ± 0.005^{b}	0.163 ± 0.001^{c}	0.131 ± 0.001^{d}
Insoluble-bound	nd	nd	nd	nd
Trigonelline				
Soluble	0.220 ± 0.007^{a}	0.191 ± 0.002^{b}	0.047 ± 0.001^{c}	0.018 ± 0.002^{d}
Insoluble-bound	nd	nd	nd	nd
Theophylline				
Soluble	0.154 ± 0.012^{aA}	0.125 ± 0.003^{bB}	0.068 ± 0.012^{cB}	0.041 ± 0.009^{dB}
Insoluble-bound	0.170 ± 0.001^{bA}	0.277 ± 0.001^{aA}	0.143 ± 0.006^{cA}	0.134 ± 0.002^{dA}

Data are presented as mean ± standard deviation ($n = 2$). Values within the same row followed by the same lower case letters are not significantly different according to Tukey's Test ($p < 0.05$). Values within the same column, referring soluble and insoluble-bound fractions of the same compound, followed by the same capital letters are not significantly different according to test t ($p < 0.05$). Abbreviations: nd, not detected.

The concentration of caffeine varied from 9.598 mg g^{-1} to 28.584 mg g^{-1}, which was also higher than values reported by other authors. Bordiga et al. (2015) evaluated dark chocolates from several countries and reported values from 0.16 mg g^{-1} to 0.35 mg g^{-1}. Alañón et al. (2016) found values between 0.5 mg g^{-1} and 3.1 mg g^{-1}. Tokusoglu and Ünal (2002) reported values in the range of 1.38 mg g^{-1} to 1.93 mg g^{-1} both in commercial chocolates.

Xanthines were detected in soluble extracts whereas theobromine and theophylline were found also in the fraction containing compounds released from their insoluble-bound form. In the case of theophylline, the range of 0.134 mg g^{-1} to 0.177 mg g^{-1}, from the insoluble-fraction represents up to 75% of the total content in the formulations (Table 2.2). Regarding the soluble fraction, also defined as crude extract, theophylline concentrations ranged from 40 µg g^{-1} to 90 µg g^{-1} in chocolate (TOKUSOGLU; ÜNAL, 2002), a value similar to the soluble fraction presented in our study. In agreement to the data obtained for phenolic acids and flavonoids, the presence of insoluble-bound lends further support on the screening of insoluble-bound bioactives from chocolate.

Nicotinic acid (0.023 mg g^{-1} to 0.402 mg g^{-1}) and trigonelline (0.018 mg g^{-1} to 0.220 mg g^{-1}) were detected only in soluble fraction (Table 2.2). Supporting the findings on phenolics, the increase of 15% of cocoa solids content (from 70 to 85%) were also important in increasing the content of nicotinic acid in the soluble form, up to 1.7- and 4.7-fold, in raw and traditional formulations, respectively. Nicotinic acid and trigonelline are not commonly studied by researchers from the chocolate field, but they may also exert important bioactivities. Nicotinic acid may contribute to reduce LDL-cholesterol and triglycerides levels. Furthermore, its antithrombotic, anti-inflammatory, and antioxidant potentials have also been reported (WHAYNE, 2014).

Likewise, trigonelline has beneficial effects in several human diseases, notably type 2 diabetes and central nervous system disorders. Its properties may also include hypoglycemic, hypolipidemic effects, neuroprotective, antimigraine, sedative, antibacterial, antiviral, and anti-tumor activities. Trigonelline may also act by improving diabetic auditory neuropathy and memory retention as well as by inhibiting platelet aggregation (ZHOU; CHAN; ZHOU, 2012).

2.3.4. Antioxidant potential

Phenolic extracts were evaluated for their antioxidant potential (Table 2.3). As expected, a higher content of cocoa solids positively affected the antioxidant capacity and the reducing power. The higher activities were observed in raw 85% sample. However, such increase was dependent on the assay, thus lending support to previous studies (DE CAMARGO et al., 2014; DE CAMARGO et al., 2015) highlighting that the antioxidant properties of food phenolics may differ according to the methods used, which may be explained by their distinct operative mechanisms.

Furthermore, it is worth to note that an increase of 15% in the cocoa solid content (from 70 to 85%) reflected in up to 1.8- and 1.6-fold higher scavenging of peroxyl radicals (ORAC assay) in the fractions containing soluble and insoluble-bound phenolics, respectively. Likewise, a significant increase (up to 2.7-fold) was observed with respect to the reducing power (FRAP assay). Therefore, it was possible to demonstrate that the antioxidant potential of chocolate was mainly influenced by their cocoa solids. Reactive oxygen species (ROS) and metal ions may induce lipid and protein oxidation thus being detrimental to biological systems (MENG et al., 2017).

A recent study (DE OLIVEIRA et al., 2017) demonstrated that an increase as 27% in total phenolics in the daily consumption was sufficient to decrease VLDL-cholesterol and triacylglycerol levels in Wistar rats. Likewise, phenolics were also able to increase serum antioxidant status and increase HDL-cholesterol in healthy adults under oxidative stress (TOSCANO et al., 2017). Low levels of HDL-cholesterol as well as high levels of triacylglycerols, non-HDL cholesterol, and plasma oxidation may have a causative role in coronary heart disease. Therefore, due to the wealth of evidences from food bioactives towards these biomarkers, the increase of phenolic compounds found in the present study may lower the risk of development of cardiometabolic diseases. In general, the fraction containing soluble compounds of traditional chocolates showed lower (circa 24% less) antioxidant potential than that of raw samples (Table 2.3).

Table 2.3 Antioxidant activity of raw and traditional chocolates with different cocoa solids contents.

	Raw 85%	**Traditional 85%**	**Raw 70%**	**Traditional 70%**
FRAP (µM Fe^{2+} g^{-1})				
Soluble	698.98 ± 65.47^{aA}	530.06 ± 83.78 ^{bA}	295.50 ± 9.26 ^{cA}	225.30 ± 39.66 ^{cA}
Insoluble-bound	372.10 ± 22.99^{bB}	455.27 ± 45.67^{aB}	136.45 ± 8.30^{dB}	233.10 ± 21.33^{cA}
ABTS (µM TE g^{-1})				
Soluble	1061.54 ± 63.75^{aA}	703.69 ± 38.2^{bA}	389.71 ± 32.83^{cA}	325.93 ± 46.53^{cA}
Insoluble-bound	156.43 ± 74.27^{abB}	263.65 ± 39.52^{aB}	69.73 ± 42.66^{bB}	73.21 ± 71.14^{bB}
DPPH (µM TE g^{-1})				
Soluble	28080.8 ± 181.20^{aA}	22214.98 ± 123.16^{bA}	10398.85 ± 129.90^{dB}	12783.68 ± 55.85^{cA}
Insoluble-bound	16049.34 ± 65.55^{cB}	17120.88 ± 263.74^{bB}	19027.55 ± 310.92^{aA}	14918.50 ± 357.31^{cA}
ORAC (µM TE g^{-1})				
Soluble	288983.42 ± 44499.20^{aA}	184867.21 ± 28362.77^{bA}	155837.33 ± 6608.15^{abA}	101422.17 ± 4233.65^{cA}
Insoluble-bound	123920.49 ± 4985.45^{aB}	110352.51 ± 5521.30^{aB}	78095.52 ± 12335.68^{bB}	71628.11 ± 11565.58^{bB}

Data are presented as mean ± standard deviation (*n* = 3). Values within the same row followed by the same lower case letters are not significantly different according to Tukey's Test ($p < 0.05$). Values within the same column, referring activity of soluble and insoluble-bound fractions determined by the same method, followed by the same capital letters are not significantly different according to test t ($p < 0.05$). Abbreviations: TE, Trolox equivalents.

According to Di Mattia et al. (2017), roasting may jeopardize the antioxidant properties of cocoa soluble phenolics. The decrease of the antioxidant properties upon roasting may occur due to oxidation of phenolic compounds and their involvement in chemical reactions such as complexation and generation of high molecular weight molecules that may reduce their solubility (BORDIGA et al., 2015). This behavior, previously discussed with respect to insoluble-bound flavonoids, reflected in their antioxidant activities. The ratio of antioxidant properties (soluble/insoluble-bound fraction) was generally higher in raw chocolates, which suggest that raw chocolates have more potential in influencing systemic antioxidant status *in vivo*. In contrast, traditional chocolates may have higher contribution than raw chocolates in modulating local gut health after colonic fermentation of insoluble-bound phenolics by human microbiota (CHANDRASEKARA; SHAHIDI, 2012; GUERGOLETTO et al., 2016).

In the soluble extracts, TPC (Fig.2.1) was positively correlated to FRAP (r = 0.9620), ABTS (r= 0.9863) and ORAC (r = 0.9890) methods, showing that evaluating total phenolics in the soluble fraction were good to predict the antioxidant potential of the samples. Many specific compounds positively correlated in the assays, suggesting the importance that they may have in the overall antioxidant activity, especially due to their correlation to reducing power (FRAP assay). It has been reported that high concentrations of ferric ions are detrimental to lipids and proteins as it causes oxidation.

Hydroxyl radicals, highly reactive ROS, are generated in the presence of hydrogen peroxide and ferrous ions through Fenton reaction (Haber–Weiss cycle), generating not only hydroxyl radicals but also ferric ions. In this cycle, a set of dynamic redox reactions take place continually, at which ferrous ions are oxidized to the ferric form and the latter one is again reduced to the ferrous form. It has been hypothesized that the ratios of ferric to ferrous ions are important for the rapid initiation of lipid peroxidation through the Fenton reaction and the ratios of 1:1–7:1 (Fe^{3+}/Fe^{2+}) are the optimum (DE CAMARGO et al., 2015).

Therefore, it is possible to suggest that high concentrations of ferric ions may cause more damage than higher concentrations of ferrous ions. The high correlation among TPC and FRAP assay may indicate that soluble phenolics from chocolate may stop or delay the Fenton reaction by reducing ferric to ferrous ions. In the insoluble-bound extracts, correlation was observed only between specific compounds and the antioxidant activity (Table S2).

The specificity of particular compounds towards several *in vitro* antioxidant activities such as the scavenging of hydroxyl and peroxyl radicals, reducing power and metal chelation, protection against ROS-induced DNA damage, and copper induced LDL-cholesterol oxidation has already been reported, thus lending support to the findings of the current study (DE CAMARGO et al., 2014; AYOUB; DE CAMARGO; SHAHIDI, 2016).

2.4. CONCLUSIONS

In general, raw chocolates contained higher concentrations of bioactive compounds than traditional ones, which explains the higher antioxidant potential of the former samples. Determination of insoluble-bound compounds was important to better understand the overall antioxidant potential of both chocolates and demonstrated that this product is a rich source of protocatechuic acid. Due to their differences in the contribution of soluble and insoluble-bound compounds, raw chocolates may have more potential in influencing systemic antioxidant status *in vivo*.

In contrast, traditional chocolates may have a greater contribution to local gut health after colonic fermentation of insoluble-bound compounds by human microbiota.

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Supplementary material

Table S1
Correlation coefficient (R^2) between compounds and antioxidant activity methods of soluble extracts.

	Protocatechuic Acid	Nicotinic Acid	Trigonelline	Catechin	Epicatechin	Epigallocatechin	Theobromine	Caffeine	Paraxanthine	Theophylline	TPC	FRAP	ABTS	DPPH	ORAC
Protocatechuic Acid	1.0000														
Nicotinic Acid	0.6254	1.0000													
Trigonelline	0.9863*	0.5778	1.0000												
Catechin	0.9691*	0.4785	0.9930*	1.0000											
Epicatechin	0.9887*	0.7346	0.9653*	0.9310	1.0000										
Epigallocatechin	0.9563*	0.5646	0.9908*	0.9872*	0.9347	1.0000									
Theobromine	0.9339	0.6817	0.9675*	0.9464	0.9369	0.9846*	1,0000								
Caffeine	0.9598*	0.6055	0.9904*	0.9808*	0.9457	0.9987*	0,9912*	1,0000							
Paraxanthine	0.9748*	0.6522	0.9927*	0.9757*	0.9686*	0.9924*	0,9896*	0,9967*	1,0000						
Theophylline	0.9875*	0.6944	0.9884*	0.9636*	0.9891*	0.9757*	0,9758*	0,9830*	0,9946*	1,0000					
TPC	0.8656	0.8390	0.8857	0.8364	0.9078	0.9052	0,9648*	0,9244	0,9348	0,9324	1,0000				
FRAP	0.9623*	0.7108	0.9788*	0.9528*	0.9687*	0.9817*	0,9943*	0,9900*	0,9961*	0,9928*	0,9620*	1,0000			
ABTS	0.9175	0.7489	0.9454	0.9131	0.9356	0.9628*	0,9949*	0,9743*	0,9777*	0,9686*	0,9863*	0,9909*	1,0000		
DPPH	0.9008	0.5646	0.9566*	0.9528*	0.8843	0.9869*	0,9869*	0,9865*	0,9721*	0,9432	0,9212	0,9684*	0,9695*	1,0000	
ORAC	0.8830	0.8885	0.8785	0.8196	0.9344	0.8797	0,9408	0,9026	0,9244	0,9373	0,9890*	0,9533*	0,9679*	0,8768	1,0000

(*) significant difference between assays ($p < 0.05$).

Table S2
Correlation coefficient (R^2) between compounds and antioxidant activity methods of insoluble-bound extracts.

	Protocatechuic Acid	Catechin	Epicatechin	Epigallocatechin	Theobromine	Theophylline	TPC	FRAP	ABTS	DPPH	ORAC
Protocatechuic Acid	1.0000										
Catechin	0.5519	1.0000									
Epicatechin	0.7203	0.9444	1.0000								
Epigallocatechin	0.4925	0.9975*	0.9237	1.0000							
Theobromine	0.7057	0.9543*	0.9056	0.9386	1.0000						
Theophylline	0.0467	0.8530	0.7108	0.8854	0.6804	1.0000					
TPC	0.5592	0.9487	0.9769*	0.9409	0.8494	0.8244	1.0000				
FRAP	0.5226	0.9872*	0.8905	0.9879*	0.9691*	0.8371	0.8861	1.0000			
ABTS	0.2779	0.9518*	0.8457	0.9695*	0.8283	0.9719*	0.148	0.9352	1.0000		
DPPH	-0.4066	-0.1944	-0.0631	-0.1762	-0.4524	0.1242	0.0863	-0.3260	-0.0072	1.0000	
ORAC	0.8154	0.8827	0.9856*	0.8511	0.8736	0.5830	0.9346	0.8206	0.7434	-0.0787	1.0000

(*) significant difference between assays ($p < 0.05$).

CAPÍTULO 3

BIOACTIVE COMPOUNDS AND ANTIOXIDANT ACTIVITY IN RAW COCOA HUSK AND NIBS FROM SOUTHERN BAHIA COCOA GEOGRAPHICAL INDICATION

(artigo submetido para avaliação na revista LWT - Food Science and Technology)

Bioactive compounds and antioxidant activity in raw cocoa husk and nibs from Southern Bahia Cocoa geographical indication

Abstract

Southern Bahia Cocoa (SBC) geographical indication was regulated to promote the traditional *Cabruca* harvest system and to standardize the cocoa production from certified producers, elevating its quality and market value. Soluble phenolics and alkaloids and those extracted from the insoluble-bound fraction of nibs and husk from SBC were identified and quantified by ultra-high performance liquid chromatography. Furthermore, the antioxidant activity was investigated with four methods (ABTS, DPPH, ORAC, and FRAP). Cocoa husk reaches the same amount of TPC than nibs. As the first screening of the soluble and insoluble-bound antioxidants in cocoa nibs and husk, this work identified in insoluble-bound fraction of cocoa husk high level of protocatechuic acid impacting the understanding of the antioxidant activity.

Keywords: Unroasted cocoa; Cabruca; UPLC-PDA; Methylxanthines; Phenolic Profile

3.1. INTRODUCTION

Geographical Indication (GI) is a type of Intellectual Property (IP) framed to agricultural products with quality derived from the environmental attributes of the defined and bounded location from which they originate. In foods, GI is widely applied and the quality and consumer preference is confirmed by literature in several products (e.g. fruits, vinegar, olive oil, fish, cheese, cider, wine, bread, pastries, honey, coffee, beer, and cocoa) (CHINNICI et al., 2009; APRILE; CAPUTO; NAGYA

JR, 2012; GODELMANN et al., 2013; LATORRE et al., 2013; RIPPON, 2014; KELLY; HOLLOWOOD; SCANNELL, 2019).

GI certified products can provide the authenticity claim to the food beyond its quality, also serving the commercial and cultural interests, encouraging tourism as well as the maintenance of traditional preparation methods (RIPON, 2014).

The Brazilian GI of Southern Bahia Cocoa (SBC) was recently regulated (BRAZIL, 2018) aiming to promote the "*Cabruca"* traditional harvesting method and elevate the quality of cocoa beans production from 84 municipalities between parallels 13º03' and 18º21'S and meridians 38º51' and 40º49'W Greenwich. This initiative links small producers to cooperatives and governmental institutions providing the required training for the certification according technical, cultural, socioeconomics and environmental aspects.

After the collapse of cocoa activity in Brazil in 1980's decade due the witches' broom disease, the SBC brings the structure to assure quality to international market and newly highlights the Brazilian cocoa with its individual characteristics elevating value and reestablishing the sustainability of the region.

In our previous work (MUDENUTI et al., 2018), raw chocolates produced with cocoa of this region demonstrated high levels of phenolics, alkaloids and antioxidant activities driving us the interest to extend the research to the feedstock and waste of chocolate productions: the beans and their husk.

Cocoa husk was initially used as fertilizer, animal feed and substrate for chemical reactions but their interesting concentrations of nutritional (e.g. dietary fibre) and bioactive compounds (e.g. phenolics) attracted attention to investigate the potential application of this undervalued by-product in food and pharmaceutical industries (BONVEHÍ; BENERÍA, 1998; BONVEHÍ; COLL, 1999; CASTILLEJO et al., 2006; KHANAHMADI et al., 2015; OKIYAMA; NAVARRO; RODRIGUES, 2017; BELŠČAK-CVITANOVIĆ et al., 2018; HERNÁNDEZ-HERNÁNDEZ et al., 2018; JOZINOVIĆ et al., 2018; SANTANA et al., 2018).;

Cocoa bean is traditionally used to produce chocolate, cocoa butter and cocoa powder. However, it has also been commercialized in small pieces, known as cocoa nibs. Cocoa nibs is applied in whole grain cereal products, confectionary preparations and sold directly to consumer for culinary purposes. With the rising interest in the highest levels of bioactive compounds, raw cocoa nibs are being marketed directly to consumer.

Cocoa and chocolate products are rich in flavanols, which possess antioxidant activity and have been reported to exert a protective effect against cardiovascular diseases, cancer and inflammatory processes in the human body. Such effects are promoted through inhibition of lipid peroxidation, platelet activation or cyclo-oxygenase and lipoxygenase activities, and enhancing levels of the endothelial-derived relaxing nitric oxide factor (LECUMBERRI et al., 2007; DI MATTIA et al., 2012).

In a recent work, de Camargo et al. (2019) showed that the inhibitory activity of phenolic acids and flavonoids towards NF-κB activation in RAW 264.7 macrophages was quite similar to the reducing power and scavenging of peroxyl radicals, as evaluated by FRAP and ORAC methods, respectively. Because lipopolysaccharide (LPS) increases ROS intracellular production, these authors suggested that the inhibition of NF-κB activation could be attributed to antioxidant pre-treatment, thus validating FRAP and ORAC as chemical colorimetric methods of biological importance.

Polyphenols are water-soluble and found in the free, esterified, and insoluble-bound forms, the latter fraction being linked to the cell walls of source materials (de CAMARGO et al, 2014). We observed before (MUDENUTI et al., 2018) that the contribution of insoluble-bound compounds in chocolate is important to the antioxidant activity understanding and were never explored in cocoa until now.

Methylxanthines consumption is anthropologically linked to the human request for foods/beverages that contained substances with added value in terms of well-being, other than just calorically. In recent years, increased attention has been brought to their dietary effects combined with relatively low toxicity: psychostimulatory activity, nervous system stimulation, anti-inflammatory action by phosphodiesterase inhibition and adenosine receptor antagonism mechanisms (MONTEIRO et al, 2016).

Consumers are becoming more and more aware of health issues, and research is needed to evaluate the effect of processing conditions on food functional properties and to optimize technological procedures that allow the right balance between health, taste and acceptability of the final product (DI MATTIA et al., 2012).

This work deals with the quantification of phenolics and alkaloids from soluble and insoluble-bound fractions and its contribution to the antioxidant activity in raw cocoa husk and nibs produced in SBC certified IG.

3.2. MATERIAL AND METHODS

3.2.1. Samples

The cocoa beans were purchased from the only two certified SBC producers IG that commercialize raw products to consumers. The geographic locations of the farms are presented in Table 3.1. The forastero cocoa type was cultivated in traditional Brazilian agroforestry. After fermenting in wood boxes, the beans were dried naturally in terraces, washed, dried in air circulating oven and vacuum sealed until the analysis. In laboratory, beans were peeled and the husk and nibs from each sample were conducted to separated extraction process.

Table 3.1 Geographic location of the cocoa farms.

Producer	City	Latitude	Longitude	Altitude (masl)[a]	Average temperature (°C)
T	Itacaré	14° 23’ 51’’ S	39° 06’ 19’’ W	120	24.5
PC	Camacan	15° 13’ 30’’ S	39° 30’ 04’’ W	200	23.6

[a] Meters above sea level.

3.2.2. Chemicals and reagents

Chromatographic standards - ascorbic acid, caffeic acid, caffeine, catechin, chlorogenic acid, epicatechin, epigallocatechin, ferulic acid, gallic acid, kaempferol, myricetin, nicotinic acid, *p*-coumaric acid, paraxanthine, protocatechuic acid, quercetin, rutin, sinapic acid, theobromine, theophylline and trigonelline - were purchased from Sigma Aldrich (St. Louis, USA). Chromatographic solvents HPLC-grade - methanol and formic acid - were purchased from LiChrosolv® (Merck**,** Darmstadt**,** Germany). The analytical grade reagents and solvents - acetone, acetic acid, calcium carbonate, ethanol, ethyl acetate, ethylic ether, ferric chloride, ferric sulfate, hexane, hydrochloric acid, methanol, potassium persulfate, potassium phosphate, and sodium hydroxide - were purchased from Anidrol (Diadema, Brazil). The reagents and standards utilized in spectrophotometric methods - 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ); 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{TM}); 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein, and Folin-Ciocalteu's reagent - were purchased from Sigma Aldrich (St. Louis, USA).

3.2.3. Extraction process

The soluble and insoluble-bound extracts were obtained according to de Camargo et al. (2015). The samples (40 g) were defatted with hexane (solid/solvent, 1:5, w/v) in a blender (Avance, Philips Walita, Varginha, Brazil) and the mixture was vacuum filtered (Whatman 40, low ash, 8 µm pore size). The procedure was repeated three times. The solids were dried at 40°C for 4 hours in an air circulating oven (MCientifica 420, Mogi das Cruzes, Brazil) and stored at -20°C. Soluble phenolics were extracted with 70% acetone (solid/solvent, 1:40, w/v) in a water bath shaker (NT-295, Nova Técnica, Piracicaba, Brazil) at 30°C for 20 min. After centrifugation at 4000 x *g* (5084R, Eppendorf AG, Hamburg, Germany), the supernatant was collected. The extraction was repeated two more times and the supernatant was combined. Acetone was evaporated under vacuum in a rotary evaporator (Model 801 Fisatom, São Paulo, Brazil) at 40°C and then lyophilized. Extracted compounds were reconstituted in HPLC-grade methanol and stored at -20 °C. The residue remaining after the extraction of soluble compounds was hydrolyzed with 4M NaOH (1:1, v/v) at 40°C for 4 hours following acidification to pH 2 with 6 M HCl. Insoluble-bound compounds released were extracted with diethyl ether and ethyl acetate (1:1, v/v). The process was repeated five times and the solvent was evaporated in a rotary evaporator at 40°C. Dried extracts were resuspended in HPLC-grade methanol.

3.2.4. Determination of bioactive compounds by ultra-high performance liquid chromatography (UHPLC)

Extracts were injected in a Waters HSS C18 column, 1.8 µm, 2.1 mm X 100 mm (Waters, Dublin, Ireland) in an UPLC® Acquity (Waters, Milford, MA, USA) coupled to a thermostatic autosampler (5°C), binary solvent manager and diode-array detector (DAD) set at 270 nm. The mobile phase was of solvent A: water and solvent B: methanol and both were acidified with formic acid (0.1% v/v). The flow rate was 0.4 $mL.min^{-1}$ following the gradient: 0 min, 5% B and 95% A; 10 min, 95% B and 5% A; 10.1 min, 5% B and 95% A. Total run time was 13 min. Limit of detection (LOD) was defined from visual inspection in 0,03 mg/L. Quantification was carried out with calibration curves from external standards with linear curves from 0,1 mg/L to 100 mg/L, defining the limit of quantification (LOQ) as 0,1 mg/L.

3.2.5. Total phenolic content (TPC)

Total phenolic contents were determined according to the procedure described by de Camargo et al. (2015). Phenolic extracts (0.5 mL) were mixed with deionized water (4 mL) and Folin-Ciocalteu’s reagent (0.5 mL). After three min, a saturated sodium carbonate solution (0.5 mL) was added and the flasks were kept in the dark for 2 hours. The absorbance was observed at 760 nm in a UV-visible spectrophotometer (Libra S22, Biochrom, Cambridge, UK). The results expressed as milligram of gallic acid equivalents per gram of chocolate.

3.2.6. Ferric reducing antioxidant potential (FRAP)

The ferric reducing antioxidant potential was carried out according to Sánchez-González, Jiménez-Escrig and Saura-Calixto (2005). A stock solution was made of 0.3 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ solution. Acetate buffer (25 mL) and TPTZ (2.5 mL) were mixed, and 2.5 mL $FeCl_3$ were added. Phenolic extracts (10 µL) were added to 900 µL of the FRAP solution and kept for 30 min in the dark. The absorbance was measured at 595 nm using a spectrophotometer (Model Libra S22, Biochrom, Cambridge, UK). The results were expressed as µmol Fe(II) per gram of chocolate.

3.2.7. DPPH radical scavenging assay

The scavenging capacity was using the DPPH radical-scavenging method described by Brand-Williams, Cuvelier and Berset (1995), with minor adaptations. Briefly, the diluted methanolic extracts (500 µL) were added to ethanol (3 mL) and the DPPH solution (0.5 mM - 300 µL). After 45 minutes in the dark, the absorbance was read at 515 nm using UV-visible spectrophotometer (Libra S22, Biochrom, Cambridge, UK). The calibration curve was made with methanolic solutions of Trolox at different concentrations (200 to 2000 µM). The DPPH scavenging capacity was expressed as µM Trolox equivalent per gram chocolate.

3.2.8. ABTS radical cation scavenging activity

The ABTS radical cation scavenging activity was conducted according to Re et al. (1999). The radical $ABTS^{\bullet+}$ was previously prepared by mixing the ABTS stock solution 7 mM (5 mL) with potassium persulfate 150 mM (88 µL). The solution was left in the dark for 16 hours. The absorbance at 734 nm was then adjusted to 0.700 nm ± 0.050 with ethanol by using a Libra S22 spectrophotometer (Biochrom, Cambridge, UK). The reaction was conducted with methanolic extracts (30 µL) and the radical $ABTS^{\bullet+}$ solution (3 mL). The standard curve was calculated with Trolox

(100 to 2000 µM) and the results expressed in µmol Trolox equivalent per gram chocolate.

3.2.9. Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed as described for Melo et al. (2015) in a microplate reader (Molecular Devices Corp., Sunnyvale, CA). Briefly, 30 µL of the standard, blank or sample were mixed directly in the microplate with 60 µL of fluorescein solution 508 mM. Subsequently, 110 µL of AAPH solution was injected in each well to initiate the reaction, following incubation at 37 °C for 15 min. The fluorescence was determined every min for 2 hours with an excitation and emission wavelengths of 485 and 528 nm, respectively. Different concentrations of Trolox were used to prepare the standard curve (12.5 to 400 µM). The results were expressed as µmol of Trolox equivalents per gram of chocolate.

3.2.10. Statistical analysis

Spectrophotometric analysis were carried out in triplicates ($n = 3$) and UPLC in duplicates ($n = 2$). One-way analysis of variance (ANOVA) and least significant difference (LSD) *post hoc* Tukey's test were used to compare the results ($P < 0.05$). Pearson correlation coefficients were also calculated. All analysis were conducted using the software Statistica 13.3 version 64 (TIBCO Software Inc., Palo Alto, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Total phenolic content (TPC)

The TPC in raw nibs and husk is presented in Fig. 3.1. Nibs T had the highest value of soluble TPC (89.80 mg GAE g^{-1}) followed by Nibs PC (55.04 mg GAE g^{-1}). These levels are similar to the TPC founded in Colombian nibs, which ranged from 44.9 to 70.0 mg GAE g^{-1} (CARRILLO; LONDOÑO-LONDOÑO; GIL, 2014) and in forastero raw nibs (48.08 mg GAE g^{-1}) evaluated by Hernández-Hernández et al. (2018) but slightly higher than that of raw nibs from Nicaragua (43 mg GAE g^{-1}) reported by Suazo, Davidov-Pardo and Arozarena (2013). The authors also roasted the sample that had the TPC decreased to 24 mg GAE g^{-1}. Vertuani et al (2014) found 30.9 mg GAE g^{-1} in Costa Rica nibs and 20.0 mg GAE g^{-1} in traditional undetermined origin nibs.

In husk samples, sample T also had the highest soluble TPC concentration (49.8 mg GAE g^{-1}), and husk PC contained 28.7 mg GAE $g^{-1.}$ It is notable that the husk reaches the same amount of TPC that many cocoa found around the world, showing that its consumption can be as beneficial as the cocoa bean itself.

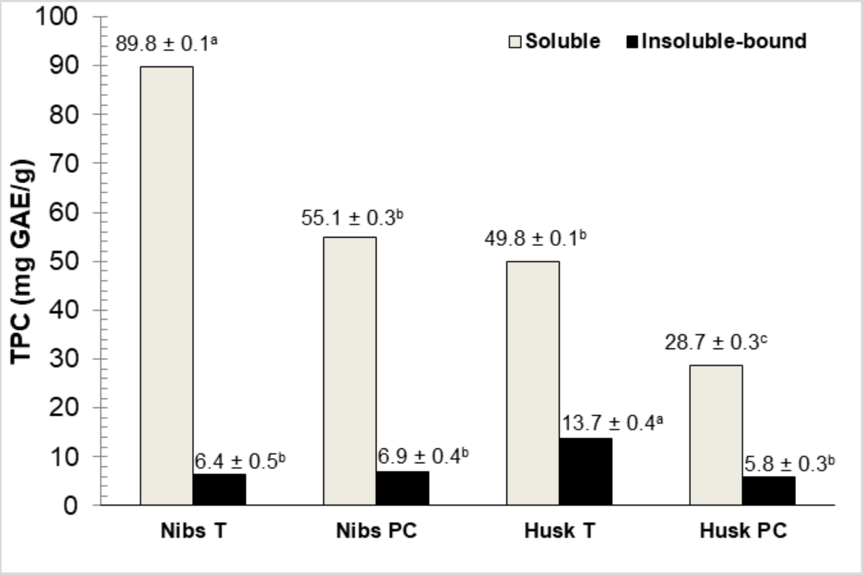

Fig. 3.1. Total phenolic content (TPC) in raw cocoa nibs and husks. Means with different letters within each fraction show difference between samples in Tukey test ($p \leq 0.05$). GAE is gallic acid equivalents.

Lecumberri et al. (2007) analyzed the TPC from roasted beans husk finding 1.3 % d.b. by Folin-Ciocalteu method. The huge difference between these results could indicate that the thermal degradation promoted by roasting on husk's polyphenol content is severe.

Mudenuti et al. (2018) noticed the rise of raw chocolate production with high-quality feedstocks such as fine cocoa from organic crops and/or GI. Thus, the raw chocolate popularization will increase the generation of raw cocoa husk by-product.

As far as we are concerned, the current work is the first to investigate the insoluble-bound phenolics in cocoa nibs and husk. Therefore, comparison with literature data is not possible. The quantities were quite similar between both nibs, but were different between the tested by-products as husk T showed 2.4-fold higher TPC in the fraction released from the insoluble-bound form.

The contribution of insoluble-bound phenolics is more significant in lower levels of soluble TPC, in Husk PV (Fig. 3.2.), as example, it consists in 17% of TPC from both fractions. Husk T presented a statically higher insoluble-bound TPC that can be driven by the crop and environmental characteristics. Carrillo, Londoño-Londoño and Gil (2014) remarked that, as any other food product, cocoa composition is influenced by changes in the environmental culture conditions, climatic and edaphological.

These findings can collaborate to the diffusion of SBC IG leading attention from consumers who seek for products naturally high in bioactive compounds and to trigger the investigation of husk utilization as food ingredient.

3.3.2 Phenolic Compounds

The characterization presented in Table 3.2 identified catechin as the major component of soluble cocoa phenols. In nibs and husk T no difference between concentrations was observed, but in sample PC, husk showed a lower concentration then the nibs. Irrespective of the sample, catechin was the main soluble monomeric flavonoid quantified.

Table 3.2. The contents of soluble and insoluble-bound phenolic compounds (mg g^{-1}).

	Nibs T	**Nibs PC**	**Husk T**	**Husk PC**
Catechin				
Soluble	33.380 ± 0.036^{aA}	27.668 ± 0.015^{aA}	29.276 ± 0.029^{aA}	14.360 ± 0.003^{bA}
Insoluble-bound	1.368 ± 0.019^{aB}	0.429 ± 0.002^{bB}	0.590 ± 0.002^{bB}	0.124 ± 0.053^{cB}
Epicatechin				
Soluble	18.333 ± 0.049^{aA}	25.121 ± 0.016^{aA}	3.674 ± 0.025^{bA}	3.731 ± 0.000^{bA}
Insoluble-bound	0.054 ± 0.001^{bB}	0.236 ± 0.008^{aB}	0.009 ± 0.000^{bB}	0.033 ± 0.003^{bB}
Epigallocatechin				
Soluble	0.819 ± 0.004^{bA}	3.177 ± 0.027^{aA}	0.6945 ± 0.008^{bA}	2.216 ± 0.005^{aA}
Insoluble-bound	nd	0.374 ± 0.001^{aB}	nd	0.297 ± 0.006^{aB}
Protocatechuic acid				
Soluble	3.204 ± 0.246^{abB}	0.805 ± 0.002^{bB}	4.058 ± 0.003^{aB}	1.337 ± 0.007^{bB}
Insoluble-bound	9.405 ± 0.246^{aA}	5.405 ± 0.010^{aA}	12.235 ± 0.031^{aA}	9.330 ± 0.022^{aA}

Data are presented as mean ± SD (*n* = 2). Values within the same row followed by the same lower case letters are not significantly different according to Tukey's Test ($p < 0.05$). Values within the same column, referring soluble and insoluble-bound fractions of the same compound, followed by the same capital letters are not significantly different according to test t ($p < 0.05$). Abbreviations: nd, not detected.

Concentrations of soluble catechin in T (33.4 and 29.2 mg g^{-1}) and PC (27.6 and 14.3 and mg g^{-1}) and epicatechin in T (18.3 and 3.67 mg g^{-1}) can be considered extremely high in comparison to literature data. Carrillo, Londoño-Londoño and Gil (2014) found the range from 0.2 to 1.3 mg $g\text{-}^{1}$ of catechin and 1.4 to 3.7 mg $g\text{-}^{1}$ of epicatechin in Colombian cocoa. Cruz et al. (2015) found the range from 0.8 to 1.6 mg $g\text{-}^{1}$ of catechin and 1.2 to 4.3 mg $g\text{-}^{1}$ of epicatechin in Southern Bahia cocoa.

These high levels of phenolic compounds in Brazilian cocoa was observed in chocolates by Cambrai et al. (2017) who found the highest levels of compounds in Brazilian samples produced with forastero type in comparison with chocolates obtained from cocoa of 12 countries and by Mudenuti et al. (2018) who studied raw chocolates produced with cocoa from this region.

There are three varieties of cocoa: criollo, forastero, and trinitario which one characterized by different chemical composition and textural and organoleptic properties. Forastero beans are dark brown, less aromatic, robust flavor, with moderate acidity, are richer in cocoa butter and have a higher content of phenolic compounds (ŻYŻELEWICZ et al., 2018). The crops in the Cabruca system are predominant from forastero type and their cultivars pará, parazinho and maranhão can blend in harvest and fermentation, some farmers conduct the process separating the cultivars or blending to achieve the characteristics that defines its production (CRUZ et al., 2015). This natural predominance of forastero type contributes to the understanding of the high levels of phenolic compounds founded in SBC IG.

The insoluble-bound catechin, epicatechin and epigallocatechin have lower concentrations in comparison to the soluble fraction and depended on the origin farm. However, regardless of the sample, the concentration of protocatechuic acid released from the insoluble-bound form was significant.

The concentrations of soluble and insoluble-bound phenolic compounds found in husk are remarkable. Polyphenols in husk can occur also due to the diffusion of these compounds to the outside of the cotyledons, enriching the material with soluble compounds (OKYIAMA; NAVARRO; RODRIGUES, 2017).

3.3.3 Alkaloids

The alkaloid profile is presented in Table 3.3. As soluble compounds, one would expect that their concentrations in insoluble-bound fraction could be low, as observed in most of our results. In soluble fraction, theobromine was the predominant compound, which, in general, was followed by caffeine and trigonelline.

Concentrations of theobromine, caffeine, nicotinic acid and paraxanthine were higher in the nibs, also as trigonelline from sample PC, but in sample T, trigonelline in husk had no significant difference from the nibs. Theophylline was the only alkaloid with higher concentrations in the husk portion.

The allelopathic theory proposes that caffeine in seed coats and falling leaves is released into the soil to inhibit germination of seeds around the parent plants (ASHIHARA; SANO; CROZIER, 2007). Theophylline is a caffeine catabolite originated by N7-demethylase action, so its presence in cocoa husk can be explicated by the same mechanism.

As observed with phenolic compounds, alkaloid levels in the both samples can be considered high when compared to literature ranging from 27.0 to 47.3 mg g^{-1} of theobromine and 2.7 to 4.7 mg g^{-1} of caffeine. Carrillo, Londoño-Londoño and Gil (2014) found the range from 7.0 to 9.6 mg g^{-1} of theobromine and 0.7 to 1.9 mg g^{-1} of caffeine in Colombian cocoa. Ramli et al. (2001) found the range from 16.2 to 17.5 mg g^{-1} of theobromine and from 2.5 to 4.1 mg g^{-1} of caffeine in Malaysian cocoa and 26.6 mg g^{-1} of theobromine and 4.9 mg g^{-1} of caffeine in cocoa from Ghana.

C. perniciosa growth is significantly inhibited by caffeine and infected stem tissue contains 7–8 times more caffeine than healthy stems, evidencing an infection defense response system in cocoa trees that enhance purine alkaloids production (ASHIHARA; SANO; CROZIER; 2007).

The higher concentrations of alkaloids in Southern Bahia cocoa can be supposed as the combination of the predominance of forastero breeds already cited as the most productive type, and their growing in natural resistance environment against *C. perniciosa* attack for several years.

Is also new the finding of trigonelline, nicotinic acid and paraxanthine in insoluble-bound fraction from cocoa, detected in samples from T farm. Insoluble trigonelline contributed to circa 10% from the total concentration, an important component that contribute to antioxidant potentials and has beneficial effects as antinflammatory, antimigraine, sedative, antibacterial, antiviral, type 2 diabetes protector, and anti-tumor activities (ZHOU; CHAN; ZHOU, 2012, WHAYNE, 2014).

Table 3.3. The contents of soluble and insoluble-bound alkaloids (mg g^{-1}) of raw cocoa nibs and husk.

	Nibs T	**Nibs PC**	**Husk T**	**Husk PC**
Theobromine				
Soluble	47.322 ± 0.047^{aA}	38.915 ± 0.019^{bA}	35.997 ± 0.011^{bA}	27.043 ± 0.031^{cA}
Insoluble-bound	0.289 ± 0.001^{aB}	0.234 ± 0.004^{aB}	0.178 ± 0.001^{aB}	0.118 ± 0.005^{bB}
Caffeine				
Soluble	4.687 ± 0.127^{a}	3.471 ± 0.083^{a}	3.023 ± 0.1640^{b}	2.718 ± 0.440^{b}
Insoluble-bound	nd	nd	nd	nd
Trigonelline				
Soluble	3.978 ± 0.086^{aA}	2.353 ± 0.01^{bA}	3.674 ± 0.025^{aA}	1.618 ± 0.001^{cA}
Insoluble-bound	0.384 ± 0.006^{aB}	0.215 ± 0.002^{bB}	0.189 ± 0.004^{bB}	0.126 ± 0.071^{bB}
Nicotinic acid				
Soluble	0.345 ± 0.015^{aA}	0.332 ± 0.010^{aA}	0.189 ± 0.017^{b}	0.114 ± 0.105^{b}
Insoluble-bound	0.002 ± 0.000^{aB}	0.002 ± 0.000^{aB}	nd	nd
Paraxanthine				
Soluble	0.305 ± 0.001^{aA}	0.321 ± 0.007^{aA}	0.119 ± 0.076^{cb}	0.111 ± 0.093^{b}
Insoluble-bound	0.045 ± 0.000^{aB}	0.034 ± 0.001^{aB}	nd	nd
Theophylline				
Soluble	0.033 ± 0.001^{aA}	0.039 ± 0.007^{aA}	0.101 ± 0.000^{aA}	0.107 ± 0.000^{aA}
Insoluble-bound	0.016 ± 0.003^{aB}	0.021 ± 0.001^{aB}	0.035 ± 0.000^{aB}	0.045 ± 0.001^{aB}

Data are presented as mean ± SD ($n = 2$). Values within the same row followed by the same lower case letters are not significantly different according to Tukey's Test ($p < 0.05$). Values within the same column, referring soluble and insoluble-bound fractions of the same compound, followed by the same capital letters are not significantly different according to test t ($p < 0.05$). Abbreviations: nd, not detected.

The caffeine content and the theobromine/caffeine ratio is been used as parameter to establish a cocoa bean classification into forastero, trinitario and criollo cocoa types according to the geographical area of the cocoa-growing regions, mainly depending on the altitude of the crops (DRAVIEUX et al., 2003, BRUNETTO et al, 2005, CARRILLO; LONDOÑO-LONDOÑO; GIL, 2014). In the present study, the correlation was examined and the results are presented in Fig. 3.2. The PC and T considered as husk and nibs separated fractions and the sum of them fitted into the proposed ratio as forastero type, which correlates to the traditional harvest of theses crops in SBC IG and its high levels of theobromine.

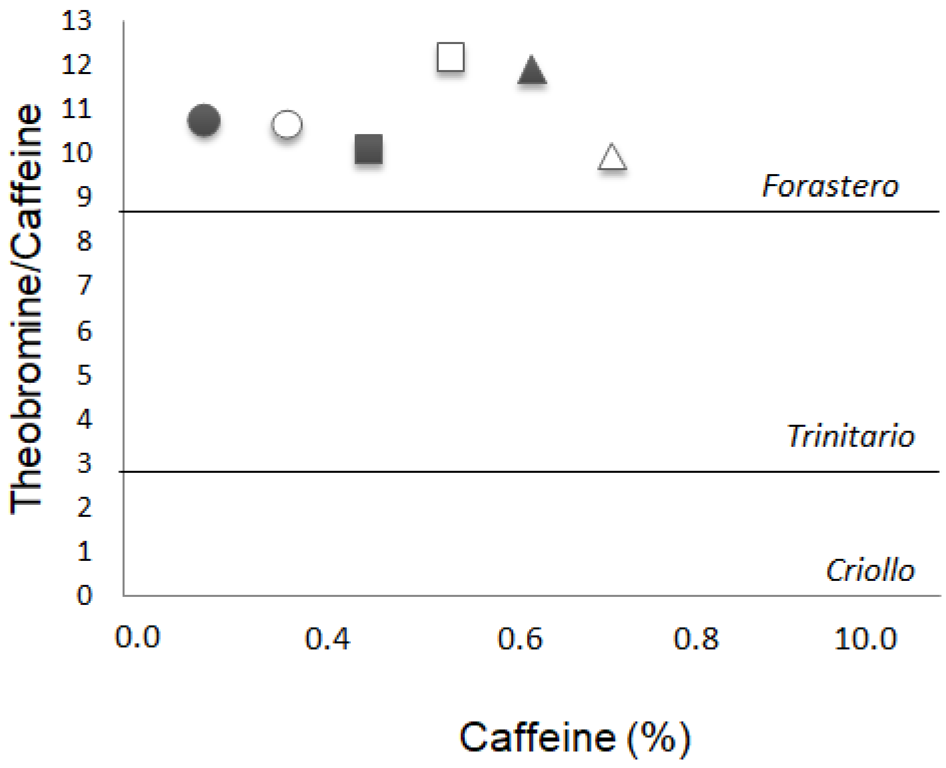

Fig. 3.2. Relationship between methylxanthine contents. ● Husk T ○ Husk PC ■Nibs T □Nibs PC ▲ Nibs + Husk T Δ Nibs + Husk PC.

Franco, Oñatibia-Astibia and Martinéz-Pinillia (2013) explains that methylxanthines are mainly acting as adenosine receptor blockers. The majority of receptor antagonists are synthetic and a significant number of antagonist are used in therapy, for instance the so-called β-adrenergic-receptor blockers that are used in diseases of the cardiovascular system. Methylxanthines are natural products that and this is probably the reason cacao, coffee, and tea, have been so successful in the lifestyle of man. Furthermore, alkaloids in cacao products have no negative effects on the health of humans because their amounts in chocolates and other processed foodstuffs containing are so low that they account for only a small proportion of the whole of the human diet (MATISSEK, 1999).

3.3.4 Antioxidant Activity

The ferric reducing activities obtained from soluble fraction vary from 350 to 1483 μM Fe^{2+} g^{-1} (Table 3.4). These levels are in agreement with the study of di Mattia et al. (2012) that found the range 713 to 930 μM Fe^{2+} g^{-1} in raw nibs from Costa Rica. Vertuani et al. (2014) found 176 μM Trolox g^{-1} in roasted beans from Costa Rica. Analyzing no origin related cocoa, Vertuani et al. (2014) found 141.66 μM Fe^{2+} g^{-1} in the nibs and Lecumberri et al. (2007) reported of 72 μM Fe^{2+} g^{-1} in the husk. Catechin (r = 0,981) and trigonelline (r = 0,960) contents correlated to FRAP assay.

Table 3.4. Antioxidant activity of raw cocoa nibs and husk.

	Nibs T	Nibs PC	Husk T	Husk PC
FRAP (μM Fe^{2+} g^{-1})				
Soluble	1483.03 ± 188.66^{aA}	953.80 ± 18.82^{aA}	1237.34 ± 47.97^{aA}	350.09 ± 5.97^{bA}
Insoluble-bound	372.10 ± 22.99^{aB}	455.27 ± 45.67^{aA}	136.45 ± 8.30^{aB}	233.10 ± 21.33^{aA}
ABTS (μM TE g^{-1})				
Soluble	2233.38 ± 89.40^{aA}	1886.96 ± 95.09^{bA}	835.53 ± 12.98^{cA}	716.09 ± 90.86^{cA}
Insoluble-bound	341.31 ± 24.96^{aB}	229.49 ± 32.38^{aB}	256.23 ± 96.08^{aB}	218.32 ± 7.31^{aA}
DPPH (μM TE g^{-1})				
Soluble	73267.20 ± 147.71^{aA}	25878.87 ± 985.13^{bA}	66425.93 ± 182.69^{aA}	22055.22 ± 193.11^{bA}
Insoluble-bound	7322.31 ± 66.49^{aB}	6259.99 ± 42.58^{aB}	6365.00 ± 231.79^{aB}	6056.14 ± 139.32^{aB}
ORAC (μM TE g^{-1})				
Soluble	$623790.30 \pm 493.08^{aA}$	$234263.31 \pm 1801.43^{bA}$	$660812.47 \pm 1982.6^{aA}$	$255315.81 \pm 2424.33^{bA}$
Insoluble-bound	60453.87 ± 207.33^{aB}	6081.17 ± 5521.30^{bB}	$61666.18 \pm 1606.06^{aB}$	6121.14 ± 234.23^{bB}

Data are presented as mean ± SD (*n* = 3). Values within the same row followed by the same lower case letters are not significantly different according to Tukey's Test ($p < 0.05$). Values within the same column, referring activity of soluble and insoluble-bound fractions determined by the same method, followed by the same capital letters are not significantly different according to test t ($p< 0.05$). Abbreviations: TE, Trolox equivalents.

These data support that IG cocoa used in fine chocolates presents higher antioxidant activity than undetermined commodity beans designated to traditional products and to the decreasing of antioxidant activity with the cocoa roasting.

DPPH and ORAC in soluble fraction showed the same behavior than FRAP, and samples from farm T had higher antioxidant activity than farm PC. DPPH assay was positively correlated only to ORAC (r = 0.980). The single effect of each compound in sample correlated to the assays explains the variance between the antioxidant activities. While trigonelline influenced DPPH (r = 0,976), epigallocatechin (r = 0,955) and protocatechuic acid (r = 0,980) showed positively relations to ORAC assay.

The ABTS differed, showing higher values in nibs (T > PC) than in the husk. Nicotinic acid, paraxanthine and theophylline were related to this assay (r = 0,968; 0,969; 0,992, respectively), as observed in Table 3.3, nicotinic acid and paraxanthine were detected only in the nibs, explaining the higher ABTS activity of this sample.

The insoluble-bound fractions had stable FRAP, ABTS and DPPH activities, without significant difference in the same origin and portion of the bean. But ORAC from samples of farm T was higher than farm PC. This result can be explained with the higher TPC (Fig. 3.1) of insoluble-bounds compounds in the husk. As discussed above, protocatechuic acid is responsible for the increase of TPC in husk T, thus demonstrating its effect in the scavenging of peroxyl radicals (ORAC assay) and the importance of its determination to understand the antioxidant mechanisms in cocoa products.

ABTS and DPPH assays were positively related (r = 0,993). Catechin (r = 0,988 to ABTS and r = 0,990 to DPPH) and trigonelline (r = 0,959 to ABTS and r = 0,978 to DPPH) related do these antioxidant mechanisms.

3.4 CONCLUSIONS

Raw nibs and husk from SBC IG can be characterized by its high levels of phenolics compounds and alkaloids and the theobromine/caffeine ratio positively allocated the product into the forastero grouping. Monomeric flavonoids and alkaloids were mainly present in the soluble fraction. However, the fraction released from the insoluble-bound form exhibited a higher concentration of protocatechuic acid, compared to all monomeric flavonoids and alkaloids, therefore being the main responsible for the antioxidant potential of this fraction. This study supports the use of raw cocoa husk as a natural source of bioactive compounds to the food industry and/or culinary purposes. Furthermore, raw cocoa husk may be used in the manufacture of nutraceuticals, thus providing a better destination to this undervalued by-product and improving the economic and sustainability aspects of cocoa production chain.

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Supplementary material

Table S1. Correlation coefficient (R^2) between quantified substances and antioxidant activity methods in soluble fraction.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	1														
2	0,515	1													
3	0,455	0,464	1												
4	0,557	0,417	0,964*	1											
5	0,928	0,666	0,349	0,369	1										
6	0,761	0,631	0,327	0,260	0,943	1									
7	0,892	0,105	0,807	0,857	0,791	0,668	1								
8	0,801	0,915	0,072	0,023	0,907	0,841	0,493	1							
9	0,614	0,978*	0,294	0,276	0,787	0,776	0,256	0,964	1						
10	0,671	0,946	0,179	0,175	0,850	0,847	0,352	0,977*	0,992*	1					
11	0,871	0,586	0,431	0,405	0,983*	0,979*	0,794	0,845	0,732	0,806	1				
12	0,981*	0,370	0,614	0,689	0,908	0,766	0,960*	0,706	0,497	0,574	0,880	1			
13	0,699	0,898	0,073	0,089	0,889	0,906	0,424	0,968*	0,969*	0,992*	0,867	0,623	1		
14	0,777	0,076	0,910	0,924	0,686	0,606	0,976*	0,330	0,093	0,202	0,723	0,883	0,292	1	
15	0,683	0,249	0,955*	0,980*	0,539	0,441	0,936	0,158	0,091	0,015	0,575	0,803	0,104	0,980*	1

1.Catechin 2.Epicatechin 3.Epigallocatechin 4.Protocatechuic acid 5.Theobromine 6.Caffeine 7.Trigonelline 8.Nicotinic acid 9.Paraxanthine 10.Theophylline 11.TPC 12.FRAP 13.ABTS 14.DPPH 15.ORAC. (*) significant different between assays ($p<0.05$).

Table S2. Correlation coefficient (R^2) between quantified substances and antioxidant activity methods in insoluble-bound fraction.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	1													
2	0,145	1												
3	0,718	0,694	1											
4	0,163	0,937	0,793	1										
5	0,867	0,365	0,343	0,301	1									
6	0,976*	0,043	0,547	0,054	0,933	1								
7	0,590	0,691	0,113	0,695	0,892	0,744	1							
8	0,719	0,539	0,039	0,569	0,934	0,851	0,981*	1						
9	0,776	0,508	0,205	0,432	0,987*	0,867	0,939	0,951*	1					
10	0,012	0,378	0,551	0,673	0,158	0,191	0,484	0,486	0,194	1				
11	0,276	0,840	0,463	0,903	0,664	0,478	0,930	0,867	0,751	0,680	1			
12	0,988*	0,270	0,754	0,248	0,789	0,950*	0,499	0,649	0,681	0,044	0,191	1		
13	0,990*	0,162	0,671	0,129	0,842	0,978*	0,596	0,734	0,748	0,136	0,308	0,993*	1	
14	0,756	0,577	0,987*	0,717	0,442	0,596	0,011	0,124	0,318	0,591	0,376	0,767	0,693	1

1.Catechin 2.Epicatechin 3.Epigallocatechin 4.Protocatechuic acid 5.Theobromine 6.Trigonelline 7.Nicotinic acid 8.Paraxanthine 9.Theophylline 10.TPC 11.FRAP 12.ABTS 13.DPPH 14.ORAC. (*) significant different between assays ($p<0.05$).

CAPÍTULO 4

METAGENOMICS PROFILING OF SPONTANEOUS COCOA BEAN FERMENTATION IN THE CERTIFIED GEOGRAPHIC INDICATION OF SOUTHERN BAHIA COCOA

(artigo submetido para avaliação na revista Food Microbiology)

Metagenomics profiling of spontaneous cocoa bean fermentation in the Geographic Indication of Southern Bahia Cocoa

Abstract

The environment of Southern Bahia belongs to Atlantic Rain Forest and allows the cocoa trees development under trees' shadows, high humidity and altitude, factors that create an agroforestry system called *Cabruca*. The Brazilian Geographical Indication (GI) of Southern Bahia Cocoa (SBC) was recently regulated aiming to promote the *Cabruca* traditional harvesting method and elevate the quality of cocoa beans production from 84 municipalities. Despite the reputation of SBC cocoa regarding its fine flavor, little is known about the microbial communities in its cocoa bean fermentations. The present study aimed at characterizing the microbiota involved in spontaneous cocoa bean fermentations of four certified producers from SBC GI. Our data showed that the microbial community is mostly composed of a consortium consisting of yeast (i.e., *Candida, Terulaspora and Saccharomyces),* lactic acid bacteria (i.e., *Lactobacillus, Leuconostoc* and *Pseudomonas*) and acetic acid bacteria (i.e., *Acetobacter* and *Gluconobacter*). Interestingly, *Botryosphaeria rhodina*, *Terulaspora delbrueckii* and *Pediococus* sp. were found in higher relative abundance than expected, and three acetic acid bacteria species were identified for the first time in cocoa bean fermentations.

Keywords: Cabruca. Yeast. Lactic acid bacteria. Acetic acid bacteria.

4.1. INTRODUCTION

Spontaneous fermentation of cocoa beans by indigenous microorganisms (mainly yeasts, lactic and acetic acid bacteria) is crucial for developing chocolate flavor precursors. Microbial communities vary in diversity, abundance, and functions along the fermentation process and uncultivable members cannot be characterized by monoculture laboratory fermentations (ILLEGHEMS et al., 2015).

In particular, this spontaneous process involves a succession of microbial activities, starting with yeasts who promote depectinization and dreinage of the cocoa pulp, degradation of carbohydrates, and production of ethanol (SCHWAN; WHEALS, 2004, DE VUYST et al., 2010, GARCIA-ARMISEN et al., 2010; ILLEGHEMS et al., 2015).

Lactic acid bacteria (LAB) become predominant after 48h from the start of the fermentation and acts converting sugars into lactic acid and some others organic acid. With the aeration increasing, acetic acid bacteria (AAB) start to convert ethanol into acetic acid in a strong exothermic reaction. This combination causes the death of the seed embryo as well as the end of fermentation. Also, this initiates biochemical changes in the beans, leading to the formation of precursor molecules for the development of a characteristic flavor and color of the beans. There is considerable overlap between the stages which finishes with the drying of the bens in sun terraces (SCHWAN; WHEALS; 2004, CAMU et al., 2007; FOWLER, 2009).

Fermentation can be conducted in heaps on the ground enclosed by banana leaves or in wooden boxes, both methods involves environmental contamination with soil, wood, handlers and tools microbiota which gave to any fermentation procedure unique microbial communities (SCHWAN; WHEALS, 2004; FOWLER, 2009).

Brazil is world-widely known for its biological diversity and there is a need for further exploring it in order to know it and to protect it. Bahia is a Brazilian State that achieved high global levels of cocoa (*Theobroma cacau*) production in 80’s whose production has declined dramatically with the groom’s which disease caused by the *Minilliophtora perniciosa* fungus attack.

After decades of intervention and natural selection of resistant crops, the cocoa production in the region returned to meet international demands and the producers seek to stablish quality and market value by promoting the proper cocoa harvest techniques traditionally develop in the region thru the century. The effort of producers and its associations resulted in the regularization of the Southern Bahia Cocoa (SBC) geographic indication (GI).

The initiative links small producers from 84 municipalities between parallels 13º03’ and 18º21’S and meridians 38º51’ and 40º49’W Greenwich to cooperatives and governmental institutions providing training required for the certification according technical, cultural, socioeconomics and environmental aspects (BRAZIL, 2018). Thus, the SBC GI brings the structure to assure quality to international market and newly highlights the Brazilian cocoa with its individual characteristics elevating value and reestablishing the sustainability of the region.

This work attempt to describe the microbiota involved in spontaneous cocoa bean fermentations of four different farms belonging to SBC IG.

4.2. MATERIALS AND METHODS

4.2.1. Samples characterization

Four producers from the SBC GI contributed to the research, allowing the visits into the farms and the sample collection. The geographic locations of the farms are presented in Table 1. All of them are producers of 'fine' cocoa beans type and the fermentation process followed their regular techniques, without any intervention from the researchers.

In order to obtain the highest number of microbial genetic information, samples were collected in the third day of fermentation (72h) when the conditions are not extreme to any present group. The cocoa beans were collected aseptically from different points from the fermentation box, placed in sterilized recipients with nutrient broth and conducted to the lab where was stored at -20°C for conservation and further metagenomics sequencing.

Table 4.1 Geographic location of the cocoa farms.

Producer	City	Latitude	Longitude	Altitude (masl)[a]	Average temperature (°C)
PV	Itacaré	14° 23' 51" S	39° 06' 19" W	120	24.7
RC	Ilhéus	14° 72' 10" S	39° 20' 47" W	100	24.5
SG	Coaraci	14° 36' 04" S	39° 37' 09" W	260	23.4
SJ	Barro Preto	14° 47' 72" S	39° 28' 83" W	200	23.5

[a] Meters above sea level

4.2.2. DNA extraction and amplification of 16S and ITS rRNA regions

The DNA from microbiota present in the beans was extracted using a Quick-DNA^{TM} Fungal/Bacterial Miniprep (Zymo Research, California, USA) assay kit according to the manufacturer's protocol. As suggested by Weisburg et al. (1991) the DNA concentration was evaluated by fluorimetry and quality was analyzed by electrophoresis (1% w/v agarose) and by polymerase chain reaction PCR using specific primers for 16S and ITS rRNA genes. PCR reactions was conducted with 20 µL final volume, with 10 µL of GoTaq® Colorless Master Mix 2x (Promega Co., Madison, USA), 0,3 µM of reverse oligonucleotide, 1µL of genomic DNA and sterile ultra-pure water to reach 20 µL.

The amplification of the 16S (V4) and ITS regions used the primers descript in Table 4.2.

Table 4.2 Oligonucleotides sequences utilized in amplification.

Primer	Oligonucleotides Sequence
16S V4-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3'
16S V4-R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3'
ITS_86-F	5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAATCATCGAATCTTTGAA-3'
ITS-4R	5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3'

The amplifications were performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, California, USA), the program for 16S (V4) region consisted of initial denaturation at 94°C for 3 min, followed by 29 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min; extension at 72°C for 1,5 min and a final extension at 72°C for 5 min. The program for ITS region consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 40s; extension at 72°C for 1 min and a final extension at 72°C for 5 min. After each sample amplification the procedure was verified in electrophoresis (2% w/v agarose) stained with UniSafe Dye 0,03% (v/v) (Uniscience, Osasco, BR).

Amplification products were purified with magnetic beads Agencourt AMPure XP (Beckman Coulter, Indianapolis, USA) and quantified with KAPA® Fast Universal kit (Merck, Darmstadt, DE) according to the supplier protocols.

4.2.3. Next-Generation sequencing and data analysis

In this step indexers were inserted in the common adapters, which are necessary for the generation of clusters and sequencing of the samples. The indexation reaction was performed following the kit protocol Nextera XT Index (Illumina, Californa, USA). The amplification program consisted of incubation at

72°C for 3 min, initial denaturation at 95°C for 30 s, followed by 12 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 30 s; extension at 72°C for 30 s and a final extension at 72°C for 5 min conducted in a Veriti 96-Well Thermal Cycler (Applied Biosystems, California, USA).

The generated libraries were purified and quantified with the same protocol descripted in the amplification step. An equimolar pool of DNA was obtained with samples normalization utilized to the NGS in MiSeq system (Illumina, Californa, USA).

Sequences with 97% similarity were assigned to the same operational taxonomic units (OTUs). Phylogenetic trees for 16S and ITS were obtained using the web tool phyloT, based on NCBI taxonomy (URL:http://phylot.biobyte.de). To estimate the microbial richness and diversity, the Chao1 and Ace estimators, Shannon and Simpson indices and Good's coverage were calculated. Rarefaction curves were plotted with base in the number of species observed and Shannon index versus the number of sequences.

4.3. RESULTS AND DISCUSSION

4.3.1 Fungal microbiota

Yeasts and the genus *Ascomycota* demonstrated diversity within the samples as presented in Table 4.3. *Saccharomyces cerevisae* dominated fungal group in SG and RC samples and was found also in PV. It is well known that *S. cerevisae is a*common and important yeast in the ethanol production.

Torulaspora delbrueckii was found in SJ, SG and PV, and dominated the yeast activity in SJ, reaching 97% of total OTUs. This species is not normally involved in cocoa beans fermentation but its presence was recorded in Ghana, Ivory

Coast, Malaysia and Ecuador (JESPERSEN et al., 2005; NIELSEN et al., 2007; PAPALEXANDRATOU et al., 2011; VISINTIN et al., 2016) but Visintin et al. (2017) had a positive influence on the analytical profile of chocolates using *T. delbrueckii* as starter culture in cocoa beans fermentation, collaborating to the ‘fine’ quality that the SBC GI wants to standardize.

Table 4.3. Identified fungi in each sample by ITS rDNA gene clone library sequencing.

Taxon	SJ	SG	PV	RC
Arthroascus schoenii	+			
Blastobotrys nivea		+		
Botryosphaeria rhodina	+		+	
Candida sp.			+	
Candida boidinii	+			
Candida jaroonii	+		+	+
Candida parapsilosis	+	+	+	
Candida pseudoaaseri		+		
Candida quercitrusa	+			
Candida sinolaborantium	+			
Cladosporium sp.		+		
Cryptococcus albidosimilis		+		
Cryptococcus flavescens	+			
Hanseniaspora uvarum	+			
Hypoxylon sp.	+			
Kazachstania hellenica			+	
Mycosphaerellaceae sp.	+			
Phanerochaete chrysosporium		+		
Pichia sp.	+		+	
Pichia burtonii			+	
Pichia jadinii				+
Preussia sp.				+
Rhodotorula mucilaginosa		+		
Saccharomyces cerevisiae		+	+	+
Schwanniomyces etchellsii		+		
Torulaspora delbrueckii	+	+	+	
Trichosporon asahii		+	+	
Wickerhamomyces sp.	+		+	

+ means presence in the sample of each farm: SJ, SG, PV and RC.

Seven different *Candida* species were identified. *C. jaroonii* and *C. parapsilosis* were present in three samples. *Pichia* genus was underrepresented in cocoa beans fermentations, probably because the Candida genus predominated.

Candida and *Pichia* species are common in cocoa fermentation in various countries and contribute to pulp degradation (ARDHANA; FLEET, 2003; JESPERSEN et al., 2005; DANIEL et al., 2009; ARANA-SÁNCHEZ et al., 2015; FERNANDEZ-MAURA et al., 2016).

Others *Ascomycota* (Table 4.3) were found in inferior abundance in the fermentation but contribute to the particular environment created in each farm.

The absence of ochratoxin producer fungi in the analyzed fermentations evidenced that the natural inhibition of the local microbiota can contribute into the mycotoxins productions in the fermentation step. In Figure 4.1 the predominant fungi are showed for each sample.

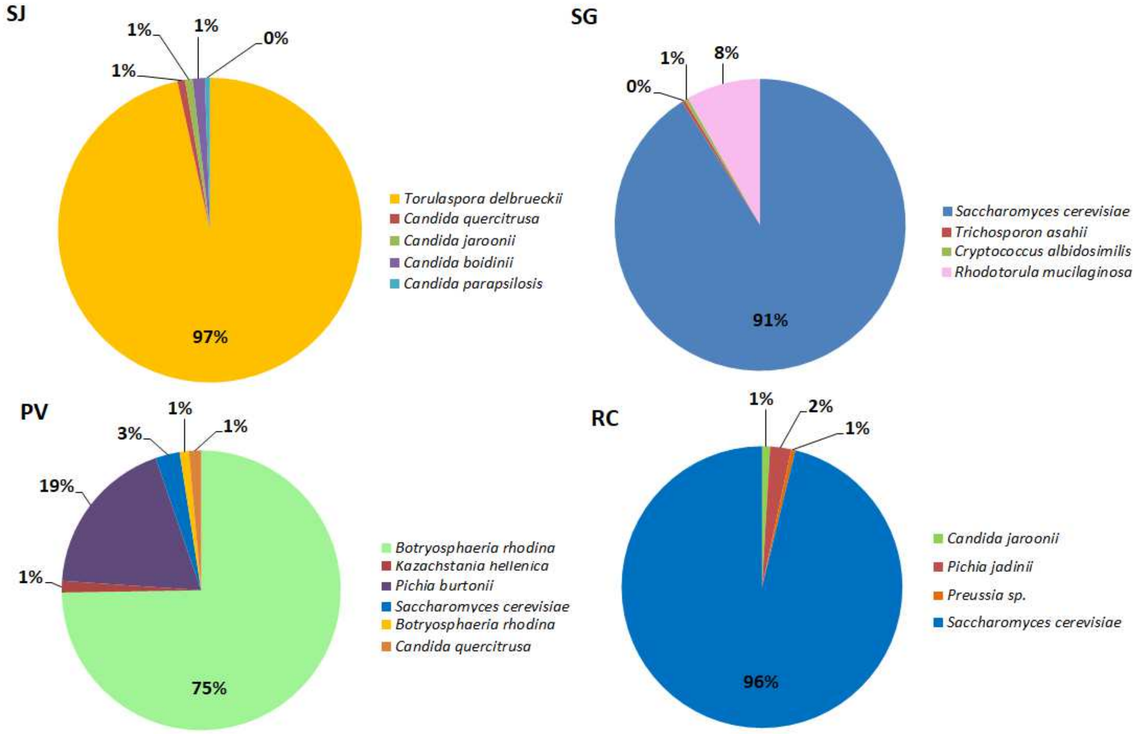

Figure 4.1. Most abundant species identified by ITS rDNA gene clone library sequencing in the cocoa beans fermentation samples.
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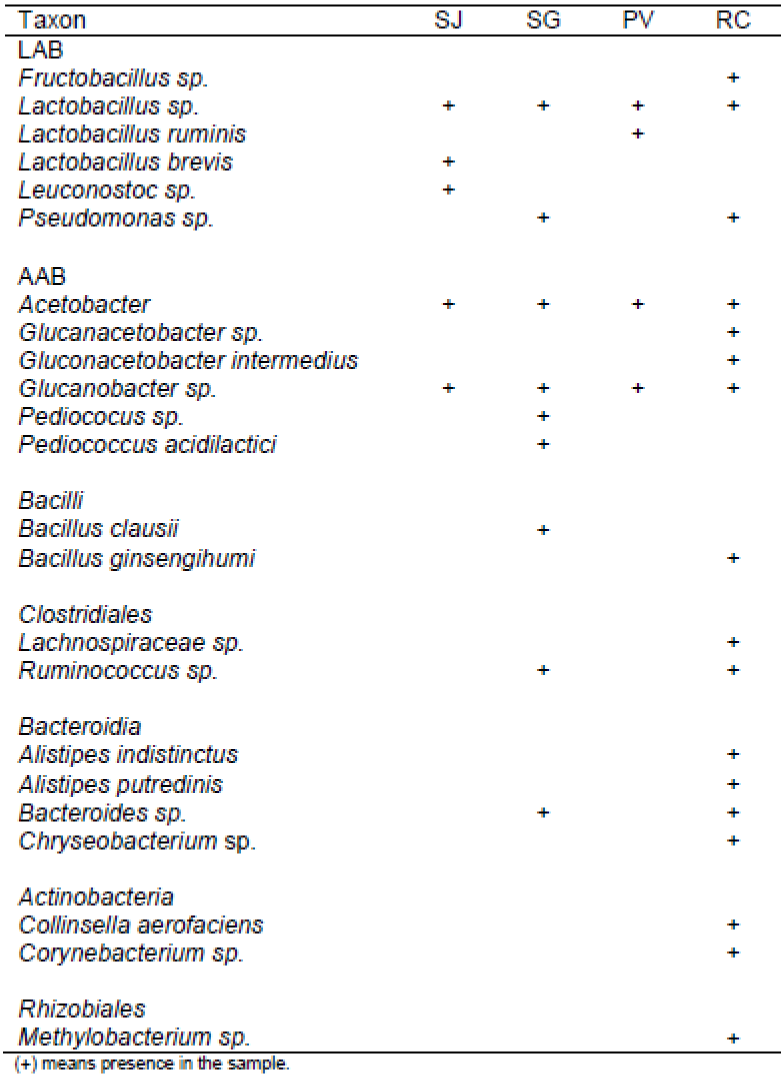
4.3.2 Bacterial microbiota

In Table 4.4 the identified bacteria in each sample by 16 S rDNA gene clone library sequencing are presented. *Lactobacillus sp.* and *Acetobacter sp.* genera were present is all samples, evidencing that these two crucial steps were conducted in the four places. The most abundant genera are showed in Figure 4.2.

Table 4.4 Identified bacteria in each sample by 16 S rDNA gene clone library sequencing.



Taxon	SJ	SG	PV	RC
LAB				
Fructobacillus sp.				+
Lactobacillus sp.	+	+	+	+
Lactobacillus ruminis			+	
Lactobacillus brevis	+			
Leuconostoc sp.	+			
Pseudomonas sp.		+		+
AAB				
Acetobacter	+	+	+	+
Glucanacetobacter sp.				+
Gluconacetobacter intermedius				+
Glucanobacter sp.	+	+	+	+
Pediococus sp.		+		
Pediococcus acidilactici		+		
Bacilli				
Bacillus clausii		+		
Bacillus ginsengihumi				+
Clostridiales				
Lachnospiraceae sp.				+
Ruminococcus sp.		+		+
Bacteroidia				
Alistipes indistinctus				+
Alistipes putredinis				+
Bacteroides sp.		+		+
Chryseobacterium sp.				+
Actinobacteria				
Collinsella aerofaciens				+
Corynebacterium sp.				+
Rhizobiales				
Methylobacterium sp.				+

(+) means presence in the sample.

Lactobacillus sp. is frequently reported as the dominant species in cocoa fermentations in Brazil, Nigeria, Ghana, Dominican Republic and Bolivia (CAMU et al., 2007; LAGUNES-GÁLVEZ et al., 2007; KOSTINEK et al., 2008; MIESCHER et al., 2016). The samples PV and SJ showed this expected dominance (Figure 4.2).

Sample SG has *Pediococcus* sp. and RC has *Gluconobacter* sp. as the dominant genus, respectively, which represents a change in the profile from LAB to AAB.

Fructobacillus pseudoficulneus is not easily identified in cocoa fermentation but was previously found in Ilhéus by Papalexandratou et al. (2011). Its presence in the sample RC (Table 4.4), from same locality (Table 4.1), shows a strong correlation to the occurrence to this municipality.

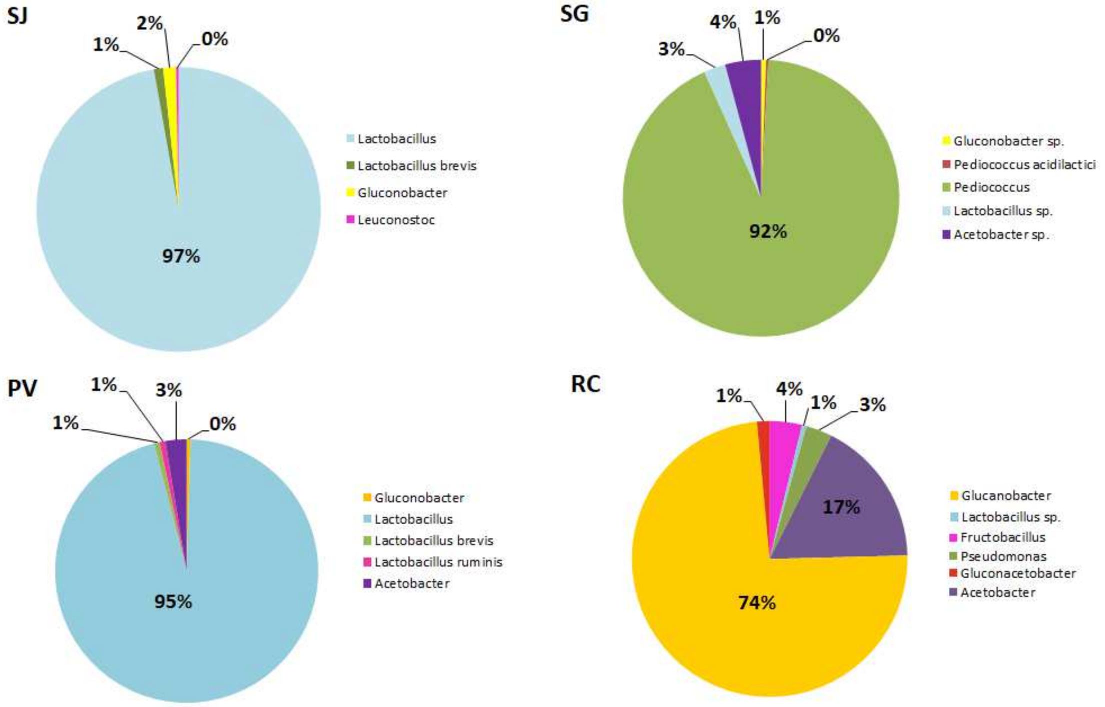


Figure 4.2. Most abundant species identified by 16 S rDNA gene clone library sequencing in the cocoa beans fermentation samples.

Bacillus species, play an essential role during cocoa fermentation due to enzyme production, such as ß-glycosidases, proteases, lipases, and amylases. These enzymes increase the production of numerous volatile compounds, including pyrazines, aldehydes, ketones, and alcohols. Additionally, *Bacillus* is a thermotolerant species and resists temperatures of 40 to 50ºC (ARDHANA; FLEET, 2003; LI et al., 2015).

Methylobacterium is related to soil and not usually found in cocoa fermentation. It is known for the production of strawberry flavor, 2,5-dimethyl-4-hydroxy-2H-furan-3-one and might contribute to the production of cocoa beans with fruitier flavors and is been proposed as a potential candidate for use as a starter culture because of it (KOUTSOMPOGERAS; KYRIACOU; ZABETAKIS, 2007) thus, their presence in the sample RC is considerably positive.

4.4 CONCLUSIONS

The results described herein are the first metagenomic study of cocoa beans fermentation from SBC IG. The microbiota responsible for the three-phase fermentation of cocoa beans was identified in all samples, indicating that the local environment and the fermentation steps conducted in the SBC GI allow the well-ordered succession of microbial groups responsible for a good quality process.

Only A*cetobacter sp., Glucanobacter sp.* and *Lactobacillus sp.* genera were common in cocoa fermentation of all localities, demonstrating the richness of microbiota composition in cocoa spontaneous fermentations. The present study suggests that most of the bacterial species implicated in the cocoa bean fermentation process from SBC GI have previously been identified and are related to flavor, color and bioactive compounds in high-quality cocoa.

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CONCLUSÃO GERAL E CONSIDERAÇÕES

Através da extração dos compostos insolúveis foi possível indentificar que o cacau e seus derivados, conhecidos por ser fonte de catequina e epicatequina, também possuem quantidades significativas de ácido protocatecuico.

Em menores quantidades, também foram encontrados teobromina, trigonelina, paraxantina, teofilina, catequina e epicatequina, na fração insolúvel, que apresenta uma ação biológica local no intestino grosso, diminuindo o pH e atuando como prebiótico, um potencial até então não relacionado ao consumo de cacau e seus derivados.

O chocolate cru apresentou maiores teores de compostos bioativos em geral, em comparação ao chocolate tradicional. Os resultados encontrados evidenciam que o cacau produzido no sul da Bahia, seguindo as técnicas de cutivo e processamento regulamentadas pela IG, apresentam elevado teor de alcaloides, compostos fenólicos e atividade antioxidante.

A comunidade microbiana se demonstrou um consórcio entre leveduras (*Candida, Terulaspora* e *Saccharomyces),* bactérias lácticas *(Lactobacillus, Leuconostoc* e *Pseudomonas)* e bactérias acéticas (*Acetobacter* e *Gluconobacter*), que estão intimamente ligadas às condições do ecossistema local e mostrando grandes diferenças entre as localidades analisadas. As leveduras predominantes em cada localidade foram *Saccharomyces cerevisae, Torulaspora delbrueckii* e *Botryosphaeria rhodina*, já as bactérias foram *Gluconobacter*, *Pediococcus* e *Lactobacillus*.

Os resultados encontrados colaboram para classificar o cacau produzido no sul da Bahia por seus altos niveis de compostos bioativos. O chocolate cru demonstrou ter maior quantidade de compostos bioativos, confirmando a suposição dos produtores, que podem contar com um novo produto de nicho específico e maior valor aregado para a região.

A testa do cacau demonstrou ter quantidade elevada de compostos bioativos, sendo um ingrediente em potencial para a indústria de alimentos.

Depois de muitos anos com a reputação de produtor de cacau de fermentação incompleta e de baixa qualidade, a análise metagenômica comprovou que a técnica de fermentação tradicional incentivada para o obtenção da IG promove as etapas sucessórias necessárias para a obtenção de amêndoas apropriadamente fermentadas.