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Acetic Acid Bacteria Analysis Using Non-Conventional Culture Media in Kombucha Fermentation

Uso de Meios de Cultura não Convencionais para Análise de Bactérias Ácido Acéticas em Fermentação de Kombucha

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Abstract

Acetic acid bacteria are involved in several spontaneous fermentation processes, such as kombucha production. Kombucha is made by fermenting a *Camellia sinensis* infusion with acetic acid bacteria and yeasts. This study aimed to evaluate six non-commercial culture media for the enumeration, isolation, and identification of acetic acid bacteria during the fermentation of black tea and green tea kombucha. The fermentation process was performed in glass flasks at 28 ± 2 °C for 15 days. Samples were collected at 0, 3, 7, 10, and 15 days. The colonies were spread-plated and subsequently identified by Sanger sequencing. We aimed to determine if non-conventional culture media could enumerate and isolate acetic acid bacteria during kombucha fermentation, which would indicate their applicability in commercial production. All evaluated media exhibited the potential for commercial use in the enumeration and isolation of acetic acid bacteria. Considering the metabolic differences in these bacteria, the combined use of mannitol yeast extract peptone and reinforced acetic acid ethanol media is recommended. Glucose yeast extract carbonate medium can be used for the qualitative selection of acetic acid-producing strains. Among the isolates obtained, *Komagataeibacter* was found to be the most abundant genus during kombucha fermentation. The use of non-conventional culture media can improve kombucha fermentation by controlling the viability of the inoculum and enhance the production and establishment of commercial culture media.

Keywords: *Komagataeibacter*. *Camellia sinensis*. Kombucha.

Resumo

Bactérias ácido acéticas estão envolvidas em vários processos de fermentação esponatânea, tais como a produção de kombucha. A bebida kombucha é obtida pela fermentação da infusão de Camellia sinensis por leveduras e bactérias ácido acéticas. Este estudo objetivou avaliar seis meios de cultura não convencionais para enumeração, isolamento e identificação de bactérias ácido acéticas durante a fermentação de kombucha de chá preto e de chá verde. O processo de fermentação foi conduzido em frascos de vidro a 28 ± 2 °C por 15 dias. Amostras foram coletadas nos tempos 0, 3, 7, 10, e 15 dias. As colônias selecionadas foram estriadas em placas e posteriormente identificadas por sequenciamento Sanger. Todos os meios de cultura analisados apresentaram potencial para uso comercial na enumeração e isolamento de bactérias ácido acéticas. Considerando as diferenças metabólicas dessas bactérias, o uso combinado dos meios (1) manitol, extrato de levedura e peptona e (2) meio reforçado de ácido acético e etanol é recomendado. O meio glicose extrato de levedura e carbonato de cálcio pode ser utilizado para seleção qualitativa de linhagens produtoras de ácido. Em relação aos isolados obtidos, Komagataeibacter é o gênero mais abundante em fermentação de kombucha. O uso de meios de cultura não convencionais pode otimizar a fermentação de kombucha pelo controle da viabilidade do inóculo e aprimorar a produção e estabelecimento de um meio de cultura comercial.

Palavras-chave: *Komagataeibacter. Camellia sinensis. Kombucha.*

1 Introduction

Acetic acid bacteria (AAB) are microorganisms belonging to the Acetobacteraceae family. They are gram-negative, strictly aerobic, non-spore-forming, catalase-positive, and oxidase-negative, with polar or peritrichous flagella (YAMADA; YUKPHAN, 2008). The optimal pH range for the multiplication of AAB is 5.0 to 6.5. They are tolerant to acidic conditions and can multiply at pH 3.0 and 4.0. The optimum growth temperature of AAB is 25–30 °C. They are ubiquitous microorganisms that can be isolated from flowers, fruits, beer, kombucha, wine, chocolate, tequila, and vinegar (YAMADA; YUKPHAN, 2008).

The Acetobacteraceae family comprises 17 genera: *Acetobacter*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Endobacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, *Kozakia*, *Neoasaia*, *Neokomagataea*, *Nguyenibacter*, *Saccharibacter*, *Swaminathania*, *Swingsia*, and *Tanticharoenia* (YAMADA, 2016). AAB nomenclature was recently modified, and the genera were reclassified. Some species previously belonging to the genera *Acetobacter* and *Gluconacetobacter* were grouped into a new genus, Komagataeibacter (MATSUSHITA; MATSUTANI, 2016). The genera most reported in kombucha fermentation are *Komagataeibacter*, followed by *Acetobacter* (ARIKAN *et al.,* 2019; CHAKRAVORTY *et al.,* 2016; COTON *et al.,* 2017; DE FILIPPIS *et al.,* 2018; GAGGÌA *et al.,* 2018; MAY *et al.,*

2019; REVA *et al.,* 2015; VILLARREAL-SOTO *et al.,* 2020).

AAB oxidise ethanol and carbohydrates to produce organic acids for energy. This process is known as oxidative metabolism and is catalysed by dehydrogenases and ubiquinones. The oxidation of ethanol to acetic acid is known as acetic fermentation (MATSUSHITA; MATSUTANI, 2016; NAKANO; EBISUYA, 2016; OKAMOTO-KAINUMA; ISHIKAWA, 2016); this fermentation may be related to the phylogeny of AAB. The genus *Gluconobacter* has a high affinity for substrates such as D-glucose, D-gluconic acid, D-sorbitol, glycerol, and ethanol. In contrast, Acetobacter and *Komagataeibacter* species have a greater affinity for ethanol than carbohydrates. Both genera can hydrolyse acetic acid and convert it into carbon dioxide and water in the absence of ethanol (MATSUSHITA; MATSUTANI, 2016). Some *Komagataeibacter* species (*K. europaeus*, *K. xylinus*, *K. intermedius*, and *K. oboediens*) are tolerant of high acetic acid concentrations and are commonly found in vinegar (NAKANO; EBISUYA, 2016).

AAB has become the focus of research related to wine deterioration (NAKANO; EBISUYA, 2016). The manipulation and use of these microorganisms outside the fermentation medium can be inefficient, affecting strain isolation, maintenance of cultures, and cultivation outside the bioreactor. Furthermore, the viability and preservation of strains present challenges (KITTELMANN *et al.,* 1989; NAKANO; EBISUYA, 2016; OLIVEIRA *et al.,* 2010).

The production of fermented beverages such as kombucha and vinegar (via the Orleans method) is an artisanal process. These fermentations are performed using an inoculum with an undefined microbiome. The absence of pure starter cultures results in difficulties in isolating, cultivating, and preserving AAB strains (KITTLEMANN *et al.,* 1989; SELLMER; TEUBER, 1991; SOKOLLEK; HAMMES, 1997; SPINOSA, 2002; OLIVEIRA *et al.,* 2010). The biotechnological and industrial potential of AAB has led to several scientific studies (SWINGS; DE LEY, 1981; CIRIGLIANO, 1982; ETANI *et al.,*1985; SOKOLLEK; HAMMES, 1997) aimed at formulating culture media and techniques for detecting, enumerating, and isolating these bacteria. AAB culture media contains ethanol, acetic acid (carbon source), minerals, and pH indicators in a double-layer medium (0.5% agar in the lower layer and 1% agar in the top layer). The cultivation requires incubation at 30 °C for 2 to 5 d (SEEARUNRUANGCHAI *et al.,*2004; YAMADA; YUKPHAN, 2008).

Kombucha is an Asian non-alcoholic, fermented beverage of a *Camellia sinensis* infusion (JAYBALAN *et al.,*2014; MARSH *et al.,* 2014). The microbial composition varies and can include AAB, yeast, and a minor quantity of lactic acid bacteria (ARIKAN *et al.,* 2019; BARBOSA *et al.,* 2021; CHAKRAVORTY *et al.,* 2016; COTON *et al.,* 2017; JAYABALAN *et al.,* 2014; MARSH *et al.,*2014; NAKANO; EBISUYA, 2016; OKAMOTO-KAINUMA; ISHIKAWA, 2016; TEOH *et al.,* 2004; VILLARREAL-SOTO *et al.,* 2020;). The inoculum used in kombucha fermentation comprises a cellulosic matrix (film) and fermented liquid. The cellulosic matrix contains AAB and yeasts immobilized on cellulose. In the fermented beverage, microorganisms are present in the free form. The process occurs statically and aerobically owing to AAB. The fermentation time described in the literature ranges from 7 to 60 d (BATTIKH *et al.,* 2012; CHEN; LIU, 2000; CHAKRAVORTY *et al.,* 2016; CVETKOVIC; MARKOV, 2005; DUTTA; PAUL, 2019; JAYABALAN *et al.,* 2007, 2014; SIEVERS *et al.,*1995; TEOH *et al.,* 2004; MALBASA *et al.,* 2008; LONCAR *et al.,* 2014; NEFFE-SKOCINSKA *et al.,* 2017; VITAS *et al.,* 2018; RAHMANI *et al.,* 2019). Kombucha is a sweet, slightly acidic, and naturally carbonated beverage. However, the origin of the inoculum can alter sensory characteristics, and drinks with longer fermentation times are less sweet and more acidic (JAYABALAN *et al.,* 2014; NEFFE-SKOCINSKA *et al.,* 2017; VITAS *et al.,* 2018).

Non-conventional culture media has been used to enumerate and identify AAB used in vinegar fermentation, such as reinforced acetic acid ethanol media (RAE), glucose yeast extract calcium carbonate (GYC) (SPINOSA, 2002; OLIVEIRA *et al.,* 2010). However, these culture media have not been used to evaluate kombucha fermentation. This study aimed to evaluate the suitability of different non-conventional culture media for the growth, enumeration, and isolation of AAB during black tea and green tea kombucha fermentation.

2 Material and Methods

2.1 Kombucha preparation: inoculum, sampling, and fermentation process

The raw materials used were black and green tea (*Camellia sinensis*) (Belo Horizonte, Minas Gerais, Brazil), and the inoculum was donated by an artisanal producer in Uberlândia, Minas Gerais, Brazil. The fermentation process was conducted in 18 glass flasks bioreactors (nine bioreactors for each type of tea) using 22.5 g of tea (black or green), 1.5 L of boiled water, 8% sucrose (120 g/L), and the inoculum (150 mL of fermented portion and 75 g of cellulosic pellicle portion). The bioreactors were covered with cotton gauze and fermented at 28 ± 2 °C for 15 d. During the fermentation process, samples (25 g of fermented portion and cellulosic pellicle mixed) were collected at 0, 3, 7, 10, and 15 d (BARBOSA *et al.,* 2020, 2021).

2.2 Enumeration and isolation of acetic acid bacteria

Six culture media (non-commercial) were used for AAB isolation (Table 1) (SPINOSA, 2002; OLIVEIRA *et al.,* 2010), with some modifications: agar double layer (1.5 and 0.9% for the superior and inferior layers, respectively) with 0.01% cycloheximide. The samples were diluted with peptone saline to 106. The aliquots were spread-plated onto

each culture medium and incubated at 30 °C for 5 days at 95% relative humidity (RH). After incubation, colonies were counted and isolated. For isolation, three colonies of each morphotype were streaked onto mannitol, yeast extract, and peptone (MYP) agar and incubated under the conditions mentioned above.

Table 1 - Composition of culture media

Components (g/L or mL/L)	Culture Media					
	AE	GYC	Moraes	MYP	RAE	Suomalainem
Glucose	5,0	40,0	10,0	-	40,0	-
Yeast extract	2,0	4,0	10,0	5,0	10,0	5,0
Peptone	3	-	10,0	3,0	10,0	-
Mannitol	-	-	-	25,0	-	-
Citric acid	-	-	-		1,5	-
Calcium carbonate	-	8,0	-	-	-	-
Dibasic sodium phosphate	-	-	-	-	2,28	-
Agar	9; 15	15	9; 15	9;15	9; 15	9; 15
Ethanol	20	-	40,0	-	20,0	15,0
Acetic acid	10	-	10,0	-	10,0	-
Acetozim DS	1,5	-	-	-	-	-
Cycloheximide	0.1	0.1	0.1	0.1	0.1	0.1

AE=Ethanol agar, GYC=glucose yeast extract calcium carbonate, MYP=mannitol yeast extract peptone, RAE=reinforced acetic acid ethanol media.
Source: resource data.

2.3 Identification of acetic acid bacteria isolates

DNA extraction, purification, and amplification were performed as previously described by Ruiz et al. (2000), with some modifications, as follows: (i) we used the GRS Genomic DNA and PCR & Gel Band Purification Kits (GRiSP Research Solutions, Porto, Portugal) for DNA extraction and purification; (ii) we used primers 16S-F (5'-GCTGGCGGCATGCTTAACACAT-3') and 16S-R (5'-GGAGGTGATCCAGCCGCAGGT-3'); (iii) for amplification, the samples were incubated at 94 °C for 5 min to denature the DNA strand, followed by 35 cycles at 94 °C/min, 58 °C/min, and 72 °C/min; and (iv) no restriction enzymes were used to digest the PCR amplification product. The isolates were identified via Sanger sequencing (16S rRNA) (RUIZ *et al.,* 2000; VAZ-MOREIRA *et al.,* 2009). The sequences obtained were subjected to BLAST analysis, and the percentage of bacterial species identified was determined using the National Center of Biotechnology Information database (http://www.ncbi.nlm.nih.gov). Sequences were deposited with NCBI SAR under BioProjects identification PRJNA665807 and PRJNA686871.

2.4 Statistical analysis

The experimental design was completely randomised in a factorial scheme with three factors (medium, tea, and time). Six levels of the medium were combined with two levels of tea measured over five times. The mixed linear model used was:

$$y = X\beta + Zg + e \quad 1/$$

where y is the data vector; β is the fixed effects vector that includes tea, medium, time, and their interactions; g is the effects vector, including random numbers of the experimental units that were measured at different times [$g \sim N(0, \psi)$]; and e is the residual vector [$e \sim N(0, \Sigma)$]; ψ and Σ are the covariance matrices of the experimental units and residuals, respectively; and X and Z are the incidence matrices of the referred effects. The variance of random effects estimation was obtained using the Restricted Maximum Likelihood method (PATTERSON; THOMPSON, 1971). Additionally, Mauchly's sphericity test (1940) was performed to verify the homogeneity of the residual covariance matrix between times. The random effects covariance matrices were assumed to be homogeneous. The Shapiro–Wilk normality test was performed for the residue within each time. Analysis of variance was performed for fixed effects, and Tukey's test was performed with 5% significance. The mixed model was fitted with the "lme4" package (BATES *et al.,* 2018), the sphericity and normality tests with the "Mauchly.test" and "Shapiro.test" functions, respectively, and Tukey's test with "agricolae" (DE MENDIBURU, F; DE MENDIBURU, M, 2020). These procedures were performed using R (2020) software (R CORE TEAM R, 2020).

3 Results and Discussion

The enumeration of AAB in different culture media during kombucha fermentation (0, 3, 7, 10, and 15 days) is shown in Table 2. The inoculum presented good viability, with average counts of 5.4 and 5.7 Log10 CFU/g for green tea and black tea kombucha, respectively. During kombucha fermentation, the microbial count varied from 4.12 Log10 CFU/g (T0) to 7.20 Log10 CFU/g (T15) for green tea kombucha and 4.11 Log10 CFU/g (T0) to 7.16 Log10 CFU/g (T15) for black tea kombucha. The time residual covariance matrix was homogeneous according to the Mauchly test, and the residuals were normal according to the Shapiro-Wilk test within each time analysed.

Table 2. Enumeration of acetic acid bacteria in different culture media during black and tea kombucha fermentation

Log_{10} UFC/g					
Green tea kombucha (time-days)					
Culture media	T0	T3	T7	T10	T15
AE	4.16Bc	5.46Bb	5.47Ba	5.82Ba	5.96Ba
RAE	4.19Bc	5.60Bb	5.42Ba	6.05Ba	5.67Ba
GYC	4.12Bc	5.52Bb	6.19Ba	6.74Ba	6.84Ba
MYP	4.63Ac	6.11Ab	7.40Aa	6.95Aa	7.20Aa
Moraes	4.47Bc	5.38Bb	5.80Ba	5.97Ba	6.25Ba
Suomaleinem	4.85Ac	6.37Ab	6.96Aa	6.63Aa	6.78Aa
Black tea kombucha (time-days)					
Culture media	T0	T3	T7	T10	T15
AE	4.51Bc	4.85Bb	5.27Ba	5.84Ba	5.50Ba
RAE	4.13Bc	5.31Bb	5.84Ba	6.44Ba	5.40Ba
GYC	4.22Bc	4.71Bb	5.39Ba	5.93Ba	6.28Ba
MYP	5.19Ac	5.65Ab	6.37Aa	6.22Aa	7.16Aa
Moraes	4.11Bc	4.92Bb	5.61Ba	6.27Ba	6.44Ba
Suomaleinem	5.49Ac	6.14Ab	6.00Aa	6.12Aa	6.97Aa

AE=ethanol agar, RAE=reinforced acetic acid ethanol media, GYC=glucose yeast extract calcium carbonate, MYP=mannitol yeast extract peptone. Equal uppercase letters in the same column and equal lowercase letters in the same row do not differ by Tukey's test at 5% ($p < 0.05$) for each kombucha type (green tea and black tea).
Source: resource data.

Harrison and Curtin (2021) evaluated the microbial composition of kombucha. The results showed a difference between the top and bottom layers of this SCOBY for both fungi and bacteria. The radial sampling position was approximately 3-log more (3.55×10^7 CFU/g versus 3.02×10^4 CFU/g, respectively) bacteria and 2-log (3.55×10^7 CFU/g versus 3.02×10^4 CFU/g, respectively) more fungi.

Wang *et al*. (2022) analyzed the cell counts of AAB in kombucha samples from New Zealand using the glucose yeast extract peptone mannitol medium (GYPM). They observed that the samples presented different counts of AAB of 4.97 ± 0.06 log CFU/L to 5.63 ± 0.02 log CFU/mL.

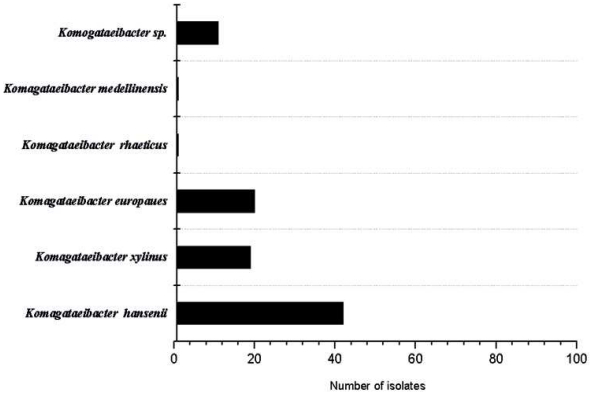
All the culture media analysed showed the capacity for growth and isolation of AAB under the examined conditions. A statistically significant difference was observed between the media and fermentation time ($p < 0.05$). MYP and Suomaleinem media showed the highest counts of AAB. A significant difference ($p < 0.05$) was observed between fermentation times; the intervals of 7, 10, and 15 days differed from the initial time points (0 and 3 days).

We observed that the cultivation of AAB in glucose, yeast extract, and calcium carbonate (GYC) media can differentiate acetic acid- or pigment-producing strains. Acid production changed the colour of the culture medium, and the calcium carbonate present in the composition was cleaved and translucent. The intensity of the colour change, from pearly to translucent, subjectively inferred acid production.

All culture media analysed showed favourable results for AAB enumeration. However, the use of RAE and MYP culture media is specifically recommended as these culture media are advantageous for counting and isolating AAB. Their compositions favour the growth of AAB, which can metabolise both carbohydrates (MYP agar) and molecules with larger carbon chains, such as ethanol and acetic acid (RAE agar). Additionally, they enable the growth of bacterial strains that produce acetic acid and tolerate high concentrations of acid in the culture medium. At the end of fermentation, it was verified that AAB counts were higher than those found in the inoculum (Table 2).

During the kombucha fermentation of green tea and black tea, 94 isolates of AAB were obtained, predominantly from *Komagataeibacter* (Figure 1).

Figure 1 - Acetic acid bacteria isolated during kombucha fermentation



Source: resource data.

The most dominant species were *Komagataeibacter rhaeticus*, *Komagataeibacter xylinus*, *Komagataeibacter europaeus*, *Komagataeibacter hansenii,* and *Komagataeibacter intermedius*.

4 Conclusion

In this study, AAB were grown and isolated during kombucha fermentation using non-conventional culture media. MYP and RAE culture media are recommended as strategies for recovering strains using different carbon sources to obtain energy. The GYC culture medium can visually indicate acetic acid-producing strains. Furthermore, using non-conventional culture media can improve kombucha fermentation by controlling the viability of the inoculum and possible establishment of commercial culture media.

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