Article

Kombucha tea as a reservoir of cellulose producing bacteria: assessing diversity among *Komagataeibacter* isolates

Salvatore La China[1], Luciana De Vero[1], Kavitha Anguluri[1], Marcello Brugnoli[1], Dhouha Mamlouk[1], Maria Gullo[1,*

[1]   Department of Life Sciences, University of Modena and Reggio Emilia, 42122 Reggio Emilia, Italy;
      salvatore.lachina@unimore.it (S.L.C.); luciana.devero@unimore.it (L.D.V.); kavitha.anguluri@unimore.it
      (K.A.); marcello.brugnoli96@gmail.com (M.B.); mamloukdhouha@gmail.com (M.D.)
*   Correspondence: maria.gullo@unimore.it (M.G.)

Abstract: Bacterial cellulose (BC) is receiving great attention due to its unique properties such as high purity, water retention capacity, high mechanical strength, and biocompatibility. However, the production of BC has been limited because of high cost and low productivity. In this light, the isolation of new BC producing bacteria and selection of high productive strains became a promising issue. Kombucha tea is a fermented beverage in which the bacteria fraction of the microbial community is composed mostly by strains belonging to the genus *Komagataeibacter*. In this study Kombucha tea production trials were performed starting from a previous batch, and bacterial isolation was conducted along cultivation time. From the whole microbial pool, 46 isolates were tested for their ability in producing BC. The obtained BC yield ranged from 0.59 g/L, for the isolate K2G36, to 23 g/L for K2G30 used as the reference strain. The genetic intraspecific diversity of the 46 isolates was investigated using two repetitive-sequence-based PCR typing methods, which are the enterobacterial repetitive intergenic consensus (ERIC) elements and the (GTG)$_5$ sequences, respectively. The results obtained using two different approaches revealed the suitability of the fingerprints techniques, showing a discrimination power, calculated as D index, of 0.94 for (GTG)$_5$ rep-PCR and 0.95 for ERIC rep-PCR. In order to improve the sensitivity of the applied method, a combined model from the two genotyping experiments was performed, allowing to discriminate among strains.

Keywords: Kombucha tea, Microbial diversity, Bacterial cellulose, *Komagataeibacter xylinus*, repetitive elements sequence-based rep-PCR, typing

1. Introduction

The attraction for BC as a natural biopolymer produced by microorganisms arises from its main functional properties and subsequent applications, especially in the biomedical, food, and engineering fields [1,2]. Bacteria able to synthesize cellulose can be considered ubiquitous since they have been found to inhabit different ecosystems. They can be isolated from environmental sources (such as soil and plants), from insects, humans, and from food sources [3].

Within the family *Acetobacteraceae*, the genus *Komagataeibacter* includes species, such as *K. xylinus*, *K. europaeus* and *K. hansenii*, that were described as cellulose producing organisms [4]. The main studied strains belong to *K. xylinus* species, which is recognized as the model organism for studying the mechanism of BC synthesis [5].

One of the most important reservoirs in which it is possible to detect strains belonging to the *Komagataeibacter* genus is Kombucha tea, a fermented beverage traditionally produced in the Asia continent, probably originated from the northeast of China [6]. Kombucha tea is characterized by a microbial community in which different microbial groups, mainly yeasts and bacteria, live in a symbiotic lifestyle [7]. Among bacteria groups, acetic acid bacteria are the main functional organisms, although also

lactic acid bacteria are found; moreover, among yeasts, strains of the genera *Saccharomyces* and *Zygosaccharomyces* are described [8]. Kombucha tea production consists of two fermentation reactions operated by yeasts and acetic acid bacteria [9]. The symbiotic relation between yeast and bacteria is established during the fermentation process, in which the yeasts are capable of hydrolysing sucrose, the main substrate for Kombucha tea production, into glucose and fructose, and then ethanol. Acetic acid bacteria use glucose, fructose and ethanol to produce gluconic acid, glucuronic acid, acetic acid, and BC [10,11].

Kombucha tea can be considered as a dynamic environment rapidly changing its composition, acting as an ecophysiological reservoir of very specialized organisms. Considering the occurrence and function of acetic acid bacteria within kombucha, previous studies described *K. xylinus* as the main species.

The ability of *K. xylinus* strains to produce BC is highly variable, making the selection of the best ones a crucial step in order to obtain candidate strains for large scale BC production. In fact, the availability of selected organisms in producing BC is a need for exploiting them as industrial machinery. The production of BC is mainly influenced by two factors. The first one is represented by the carbon sources used in the medium [12,13]; pure carbon sources could increase the production cost, making the process unsustainable. The second issue is related to the ability of the organism in growing and producing BC in the selected conditions [14]. Some studies described the abilities of strains belonging to the *Komagataeibacter* genus in producing BC at different yields [4].

In this work, acetic acid bacteria were collected from liquid and pellicle fraction of Kombucha tea samples. Totally 46 isolates were screened for their ability in producing BC and for the main phenotypic characteristics. Moreover, they were typed by applying fingerprint analysis based on (GTG)$_5$ repetitive elements and Enterobacterial Repetitive Intragenic Consensus (ERIC) elements) in order to assay the intraspecific genetic diversity.

2. Materials and Methods

2.1. Kombucha preparation, sampling and physico-chemical determinations

Duplicates of Kombucha tea from black and green teas, referred to green tea kombucha (GTK) and black tea Kombucha (BTK), were prepared and inoculated according to Jayabalan et al. [15]. Briefly, sucrose 10% (w/v) was added to demineralized water and allowed to boil. Afterwards, 1.2 % (w/v) black or green tea (Les Jardins du thé, Office Tunisien du Commerce, Tunisia) was added and allowed to infuse for about 5 min. After filtration through a sterile sieve, 200 ml of teas were poured into 500 ml glass jars previously sterilized at +121°C for 20 min. The cool tea broth was inoculated aseptically with both 3% (w/v) pellicle fraction fragments and 10% (v/v) of fermented liquid broth from a previous preculture (P) imported from the National Institute of Applied Sciences and Technology (INSAT), Tunisia. The jar was covered with sterile gauze. Fermentation was carried out in the dark for 12 days.

Cell morphology and direct observation of samples were carried out using optical microscopy at 100X of magnification, using C. Zeiss microscope apparatus (Axiolab).

Titratable acidity was determined by neutralizing samples at pH 7.2 with 0.1 N of NaOH (it was assumed that all media acidity was due to acetic acid) and expressed as % (wt/wt); pH was measured by CRISON, MicropH, 2002 pHmeter. Ethanol, expressed as % (v/v), was checked by densitometry measure using a hydrostatic balance after distillation.

2.2. Plating and isolation

Plating was performed immediately after sampling. Therefore, 10 ml of 0.1% (w/v) peptone water was added to 1 g of black or green kombucha pellicle in a sterile stomacher bag that was vigorously shaken for 5 min in a laboratory blender STOMACHER® 400 (Seward, England) to obtain a uniform homogenate. Samples (1 ml each) of the

homogenate and liquid phase were 10-fold serially diluted in 0.1% (w/v) peptone water, from which aliquots (0.1 ml) were plated on GYC medium (10 g/L yeast extract, 50 g/L glucose, 15 g/L CaCO3, 9 g/L bacteriological agar) and ACB agar medium (30 g/L yeast extract, 1 ml of a 22 g/L bromocresol green solution, 20 g/L bacteriological agar). The media were previously autoclaved at 121 °C for 15 min and after 20 ml/L of filtered ethanol 95 % (v/v) was added aseptically to ACB medium. 0.2 ml of cycloheximide hydroalcoholic solution at 25 % m/v were added directly to petri dishes.

Plates were incubated at +28°C for 72-120 hours. Colonies were picked up from a suitable dilution of each sample on GYC and ACB agar media, grown for 3-5 days at +28°C and purified through subculturing and plating. Pure kombucha isolates were named and strains were cultivated on the corresponding isolation medium for 3-5 days at +28°C [16]. Long-term preservation methods of the strains were performed according to the standard procedures of the Microbial Resource Research Infrastructure-Italian Joint Research Unit (MIRRI-IT) [17]. Specifically, a seed lot of the strains was stored at -80 °C adding glycerol 50 % (v/v) to the liquid media. The strains with a significant cellulose production were deposited in the University of Modena and Reggio Emilia (UNIMORE) Microbial Culture Collection (UMCC, www.umcc.unimore.it).

2.3. Phenotypic characterization

Strains were phenotypically characterized by determination of cell morphology, gram staining, catalase activity, KOH test, consumption of calcium carbonate and oxidation of ethanol and acetic acid on ACB agar medium, as previously reported in Gullo et al. [18].

The BC production test was carried out by collecting the pellicles and boiling them in 4 ml of 5.0% NaOH solution for 2 hours. Cellulose was confirmed when the pellicle did not dissolve after boiling [19]. Strains *K. xylinus* (DSM 2004) and *G. oxydans* (DSM 3503) were used as positive and negative controls, respectively. Tests were conducted in triplicate.

The amount of BC was estimated as reported in Gullo et al. [20]. Briefly, pellicles were washed with distilled water and then treated with 1% NaOH at 90°C for 30 min. Treated BC was washed twice with distilled water, the pH of the residual water was determined; the washing step was repeated until reaching neutral pH. Drying was conducted at 80°C. BC weight was measured by analytical balance (Gibertini E42S) [21]. The yield of BC produced was expressed in grams of dried BC per liter of broth.

2.4. Genomic DNA extraction, RFLP and amplification of (GTG)₅/REP-PCR and ERIC elements

Genomic DNA extraction was performed on liquid cultures, which were previously incubated for 72 hrs at +28°C. Flasks were vigorously shaken to remove the embedded cells from BC, the liquid medium was transferred to a sterile tube and centrifuged at 12000 g for 5 min. gDNA was extracted using the method previously proposed by Gullo et al. [22]. gDNA was checked by 3 % gel agarose in 1X TBE buffer and quantified by spectrophotometric measure (NanoDrop ND-1000). Band sizes were determined using a 100 bp DNA ladder (Invitrogen).

RFLP analysis on the full-length 16S rRNA gene was performed using *Rsa*I and *Alu*I endonucleases (Fermentas, Hanover, ND, USA), incubating at +37°C for 2h. Restriction reaction was stopped by incubation at 80°C for 20 min. The fragments were checked on 3 % gel agarose in 1X TBE buffer and sizes were determined using 100 bp DNA ladder (Invitrogen).

(GTG)₅ rep-PCR fingerprinting was carried out according to the method of Versalovic et al. [23], with some minor modifications. Isolates were subjected to REP-PCR with a single oligonucleotide GTG₅ (5'- GTGGTGGTGGTGGTG-3'). Samples were incubated for 5 min at 94°C and then cycled 35 times at 94°C for 30 s, 40 °C for 1 min, and 72 °C for 4 min. The samples were incubated for 7 min at 72 °C for final extension and kept at 4°C. ERIC elements were initially amplified according to Versalovic et al. [24] using the

primers ERIC 1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC 2 (5'-AAGTAAGTGGGGTGAGCG-3'). Samples were incubated for 5 min at 94°C and then cycled 30 times at 94°C for 30 s, 57°C for 30 s, and 65 °C for 4 min. The final extension was performed at 65°C at 8 min and kept at 4°C until tested. The reproducibility of ERIC/PCR was also tested by amplifying gDNA from randomly chosen strains several times. For both rep-PCR, pattern band lengths were determined by comparison against a 100 bp plus DNA ladder for the smallest bands and by 1 Kbp DNA ladder for the largest bands (Takara Bio, Inc., Japan).

2.5. Statistics and clustering analysis of (GTG)$_5$ and ERIC rep-PCR patterns

Patterns obtained from (GTG)$_5$ and ERIC rep-PCR runs were imported into BioNumerics tool, package version 8. The cluster analysis was performed calculating Dice coefficients using a tolerance of 1% and optimization of 1%. Clustering based on Dice coefficients was performed using the unpaired group method with arithmetic average (UPGMA) method. For each fingerprint assay, the cluster cut-off analysis was applied to define the most reliable clusters. The cophenetic correlation index was used as a statistical tool to evaluate the quality of branches. For each of the genotyping experiments, the discriminatory index (D), expressed as the probability that two strains consecutively taken from a sample would be placed into different clades, was calculated as described by Hunter and Gaston [25].

3. Results

3.1. Kombucha characteristics and isolated strains

Within 3 days of cultivation in 600 mL beakers, the two Kombucha tea samples, GTK and BTK, appeared with a thin EPS layer that became thicker along time (from 2-3 mm to 10 mm at the end of the cultivation period). Optical microscopy observation showed a high number of free bacterial and yeast cells, as well as aggregates of cells within the matrix, making cell counting uncertain and not enlightening even from the first day of fermentation.

Titratable acidity reached a maximum of 12 g/L at the end of fermentation in the black kombucha trial (GTK) and 6 g/L in the green one (BTK). pH dropped from approximately 3.7 until 2.75 for both samples, as a result of acid formation. Although no inhibition compounds were determined in this study, the difference in the final amount of acetic acid of GTK and BTK samples can be due to the occurrence of more antibacterial compounds in GTK [26]. Ethanol was nearly 0 at the beginning of the cultivation time and reached 0.28 % (v/v) and 0.30 % (v/v) at the final time, for samples GTK and BTK, respectively. The low value of observed ethanol is in agreement with the gradual increase of acetic acid during cultivation time.

The isolation was performed from preculture (P) and samples at 0, 6, and 12 days of cultivation. A total of 122 isolates were selected based on the colony and/or cellular morphologies. Microscopically, presumptive acetic acid bacteria cells appeared in single or grouped pairs or short chains,gram-negative, catalase-positive, and KOH positive. A total of 46 isolates, which oxidized both ethanol and acetic acid, as confirmed by the ethanol chalk-ethanol test on GYC and the colorimetric assay on ACB, and produced a well-defined cellulose layer and or formed both cellulose layer and cellulose particles dispersed in the surrounding liquid, were considered in this study (Table 1).

Table 1 Features of 46 isolates from Kombucha tea used in this study. Site of isolation and RFLP analysis. In bold are reported the strains deposited in UMCC collection; in brakets is the accession number assigned.

| Isolate	Sample	Time	BC (g/L)	Colony morphology	Liquid growth	*Alu*I (pb)	Group	*Rsa*I (bp)	Group	Reference
K1A18	liquid	6	4.0448±0.0008	green cellulosic	cellulose layer	-	-	120, 400, 425, 550	A	This study
K1A34	liquid	12	5.6214±0.0010	green with light cellulosic edge	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K1A6	liquid	0	3.0024±0.0009	cellulosic small	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K1A7	liquid	0	1.5886±0.0004	cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	This study
K1A8	liquid	0	2.5886±0.0002	cellulosic	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K1A9	liquid	0	3.4014±0.0004	green cellulosic	cellulose layer	-	-	-	-	This study
K1G2 (=UMCC 2964)	liquid	P	4.7895±0.0014	white cellulosic edge	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K1G22	liquid	12	0.7057±0.0003	cellulose light	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K1G23	liquid	12	4.8122±0.0003	dark cellulose	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	[20]
K1G24	liquid	12	4.1210±0.0001	white cellulose	only cellulose deposit	-	-	120, 400, 425, 550	A	[20]
K1G3	liquid	P	2.1857±0.0052	light beige	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	[20]
K1G4	liquid	0	1.1738±0.0032	cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K1G5	liquid	0	4.9590±0.0027	cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K1G6	liquid	0	5.3676±0.0025	white cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2A10 (= UMCC 2965)	liquid	0	5.0405±0.0008	cellulosic green small	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study

K2A28	liquid	6	5.8962±0.0007	green cellulosic	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K2A32	pellicle	6	5.6143±0.0001	green cellulosic	cellulose layer	-	-	-	-	This study
K2A33	liquid	6	0.6414±0.0005	green cellulosic	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K2A44	liquid	12	5.1971±0.0002	small green	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	This study
K2A45	pellicle	12	5.2133±0.0004	light edge	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K2A46	liquid	12	5.7833±0.0001	dark center light edge	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K2A47	pellicle	12	5.8019±0.0001	blue medium displayed	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	This study
K2A7	liquid	0	4.6076±0.0007	green cellulosic small	cellulose layer	150, 450, 800	1	400, 450, 550	B	This study
K2A8	liquid	0	4.4781±0.0004	green cellulosic small	cellulose layer	-	-	-	-	This study
K2G1	liquid	P	6.8843±0.0004	cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	This study
K2G10	liquid	0	4.9752±0.0016	cellulosic small	cellulose layer/deposit	150, 450, 800	1	400, 450, 550	B	[20]
K2G11	liquid	0	1.0638±0.0003	white cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G12	liquid	0	6.2805±0.0001	small cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G14	liquid	0	7.7414±0.0005	white cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G15	liquid	0	7.5448±0.0001	creamy	cellulose layer/deposit	150, 450, 800	1	400, 450, 550	A	[20]
K2G2	pellicle	P	6.2548±0.0004		cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G29 (= UMCC 2967)	pellicle	6	7.5848±0.0002	cellulosic	cellulose layer	150, 450, 800	1	400, 450, 550	B	[20]

K2G30	pellicle	6	23.1829±0.0001	cellulosic	cellulose layer/deposit	150, 450, 800	1	400, 450, 550	B	[20]
K2G31	pellicle	6	1.1267±0.0007	cellulosic	cellulose layer/deposit	-	-	-	-	[20]
K2G32 (= UMCC 2968)	liquid	6	9.9976±0.0003	cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G34	liquid	6	6.6614±0.0001	cellulosic	cellulose layer	-	-	-	-	[20]
K2G35	liquid	6	1.9314±0.0005	cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G36	liquid	6	0.5929±0.0004	yellow cellulosic	cellulose layer	150, 450, 800	1	400, 450, 550	B	[20]
K2G38 (= UMCC 2969)	liquid	6	9.6957±0.0009	cellulosic	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G39 (= UMCC 2970)	liquid	6	10.3957±0.0001	cellulosic	cellulose	150, 450, 800	1	400, 450, 550	B	[20]
K2G41 (=UMCC 2971)	liquid	6	9.1443±0.0003	cellulosic	cellulose layer	150, 450, 800	1	400, 450, 550	B	[20]
K2G44 (= UMCC 2972)	pellicle	12	5.9500±0.0005	dark brown cellulose	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G46	liquid	12	9.1195±0.0007	brown cellulose	cellulose layer/deposit	-	-	-	-	[20]
K2G5 (=UMCC 2966)	liquid	P	8.0605±0.0003	white cellulosic	cellulose layer	-	-	120, 400, 425, 550	A	[20]
K2G6	liquid	P	7.7533±0.0003	white cellulosic	cellulose layer/deposit	150, 450, 800	1	120, 400, 425, 550	A	[20]
K2G8	liquid	0	6.8343±0.0002	white cellulosic	cellulose layer	150, 450, 800	1	120, 400, 425, 550	A	[20]

(-) not detected

Qualitative cellulose tests confirmed their ability in producing BC. Significant macroscopic diversity in the cellulosic material was observed coupled with high variability in terms of amount of BC, as reported in Figure 1. The strains with a BC production at least higher than 4 g/L were deposited in UMCC culture collection with the code reported in Table 1.
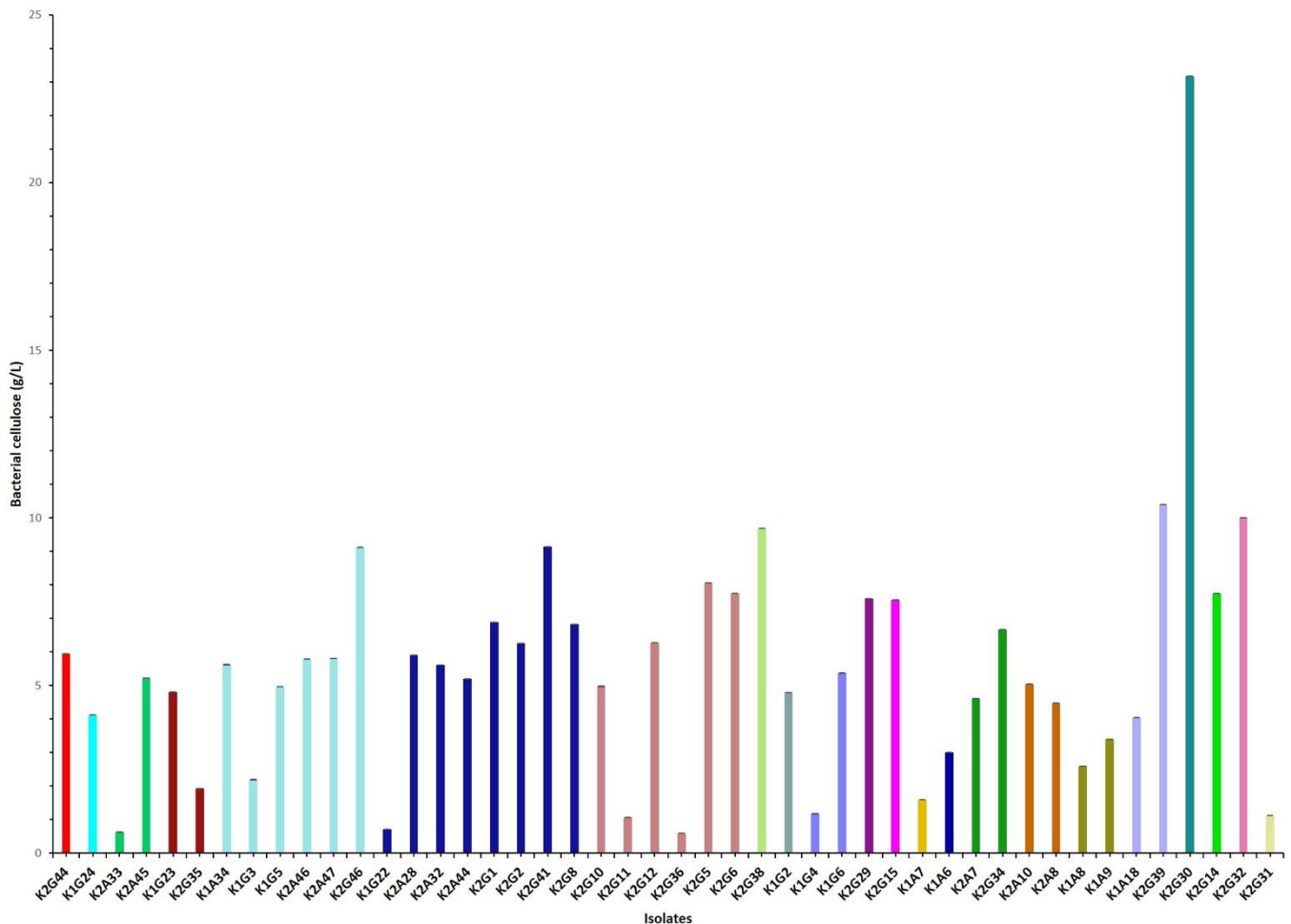
Figure 1. Bacterial cellulose yields of selected strains isolated from Kombucha tea. Bar color was performed according to the combined pattern analysis from (GTG)$_5$ and ERIC rep-PCR as represented in Figure 3.

3.2. Diversity of acetic acid bacteria from Kombucha tea

The RFLP analysis on 16S rRNA, using *Rsa*I and *Alu*I restriction endonucleases, was conducted as a preliminary test to investigate the diversity of isolates recovered from Kombucha tea samples. The mapping analysis based on the size of the fragments obtained by *Rsa*I and *Alu*I showed high homogeneous results, indicating the isolates as members of the same species. This evidence was in agreement with a previous study [16] which grouped *Komagataeibacter* strains according to *Rsa*I and *Alu*I restriction enzymes and observed the similarity of the pattern of *K. xylinus* DSM 2004 with *Komagataeibacter* strains investigated in this study. The low variability of the fingerprinting pattern is also consistent with previous studies on the high similarity of the 16S rRNA gene among the *Komagataeibacter* genus and especially within the *K. xylinus* species [27,28].

From the genotyping of strains performed by using $(GTG)_5$ and ERIC fingerprinting techniques, two dendrograms were generated (Figure 2). Based on the cluster cut-off analysis, profiles obtained using the amplification of interspersed tandem repeats GTG (Figure 2, a) discriminated against the strains considering a minimum percentage of similarity of 27.9%. A total of 5 major clades were created with a discrimination power of 0.42, calculated using the D index (Simpson index) [25]. In order to improve the discrimination power of the analysis, we considered a minimum percentage of similarity of 70%. At this percentage of similarity, the resulted discrimination power was 0.94, defining 20 different biotypes. Among the detected clades, 8 of them included just one isolate while 7 were formed by 2 isolates. It was possible to distinguish two major clades, of which one included 9 isolates (clade 18) and one of 5 isolates (clade 19). The two reference strains, represented by K1G4 (=UMCC 2947) and K2G30 (=UMCC 2756) clustered into two different clades, represented by clades 19 and 7, respectively. The similarity percentage among the two references was 40.2%.

The phylogenetic tree obtained from the digitized pattern profile using ERIC rep-PCR was represented in Figure 2 (b). Based on the clustering cut-off analysis, ERIC was able to discriminate using a minimum similarity percentage of 40%, identifying 5 major clades, as in the case of $(GTG)_5$. The clustering cut-off analysis discriminated a total of 19 biotypes, with a discrimination power of 0.95, slightly higher compared to $(GTG)_5$. A total of 6 clades were represented by just one isolate, while the remaining isolates were well distributed among the detected clades. Three clades were represented by 5 isolates (clade 7, 16, and 17) and 2 clusters by four isolates (clade 8 and 9). The remaining clades were represented by three and two isolates. The number of detected clades was not so different compared to $(GTG)_5$ and, also in this case, the reference strains (K1G4 and K2G30) were found to be separated into two different groups (clade 15 for K1G4 and clade 12 for K2G30). The similarity of Dice indexes based on the patterns obtained from ERIC rep-PCR was 56.7%, quite similar to that obtained using $(GTG)_5$ rep-PCR (40.2%).
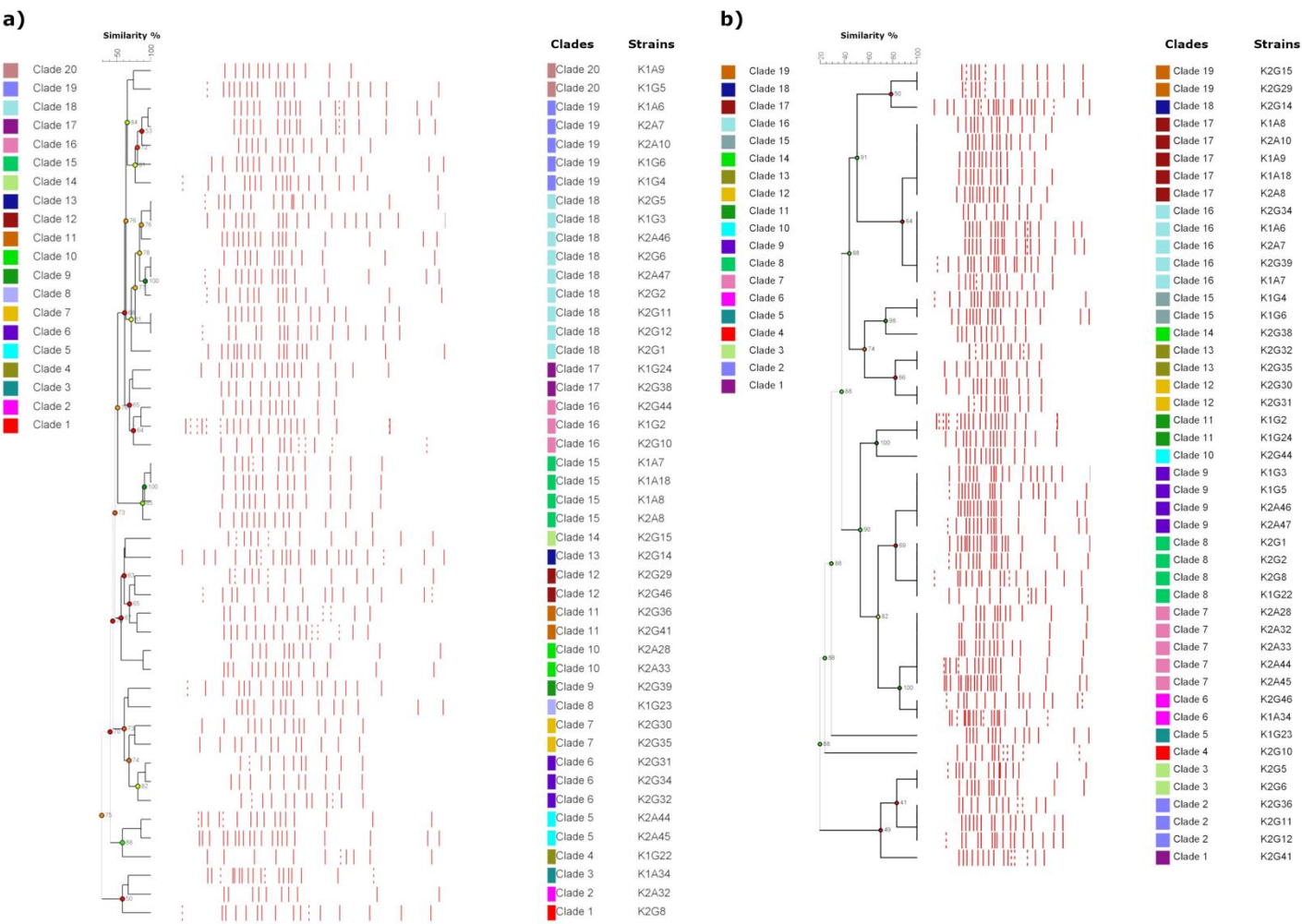
Figure 2 Dendrograms obtained from UPGMA analysis, using Dice's coefficient, of the digitized patterns obtained from (GTG)₅ rep-PCR (a) and ERIC rep-PCR (b). The similarity threshold for biotypes discrimination was 70%. The cophenet coefficient was represented by numbers and dots colored as red-orange-yellow-green, based on the branch quality.

3.3. Improving the fingerprinting of Komagataeibacter strains

In order to improve the sensitivity of the intraspecific discrimination, the data obtained from both rep-PCR assays were combined. The clustering analysis is reported in Figure 3. Based on the cluster cut-off analysis, the combination of (GTG)₅ and ERIC rep-PCR allowed discriminating isolates grouping them into 22 clades, considering a similarity percentage threshold of 94%. The discrimination power calculated as Simpson index was 0.94, mining a high intraspecies diversity. Most of the isolates were grouped into three clusters (clade 5, 6, and 7) representing 43.5% of the isolates (N=20). The remaining isolates (N=26) were dispersed in 19 clades, of which most of them consist of just one isolate (N=12). The reference strains were clustered into two different clades, as observed in the case of both (GTG)₅ and ERIC rep-PCR. Clade 10, in which K1G4 was clustered with K1G6, and clade 19, where K2G30 clustered alone, had a similarity percentage of Dice index of 84.9%, higher considering the separated results from (GTG)₅ and ERIC rep-PCR. This result is in agreement with the meaning of the combined analysis, since the two reference strains belong to the species of *K. xylinus*, as previously stated [12,29].
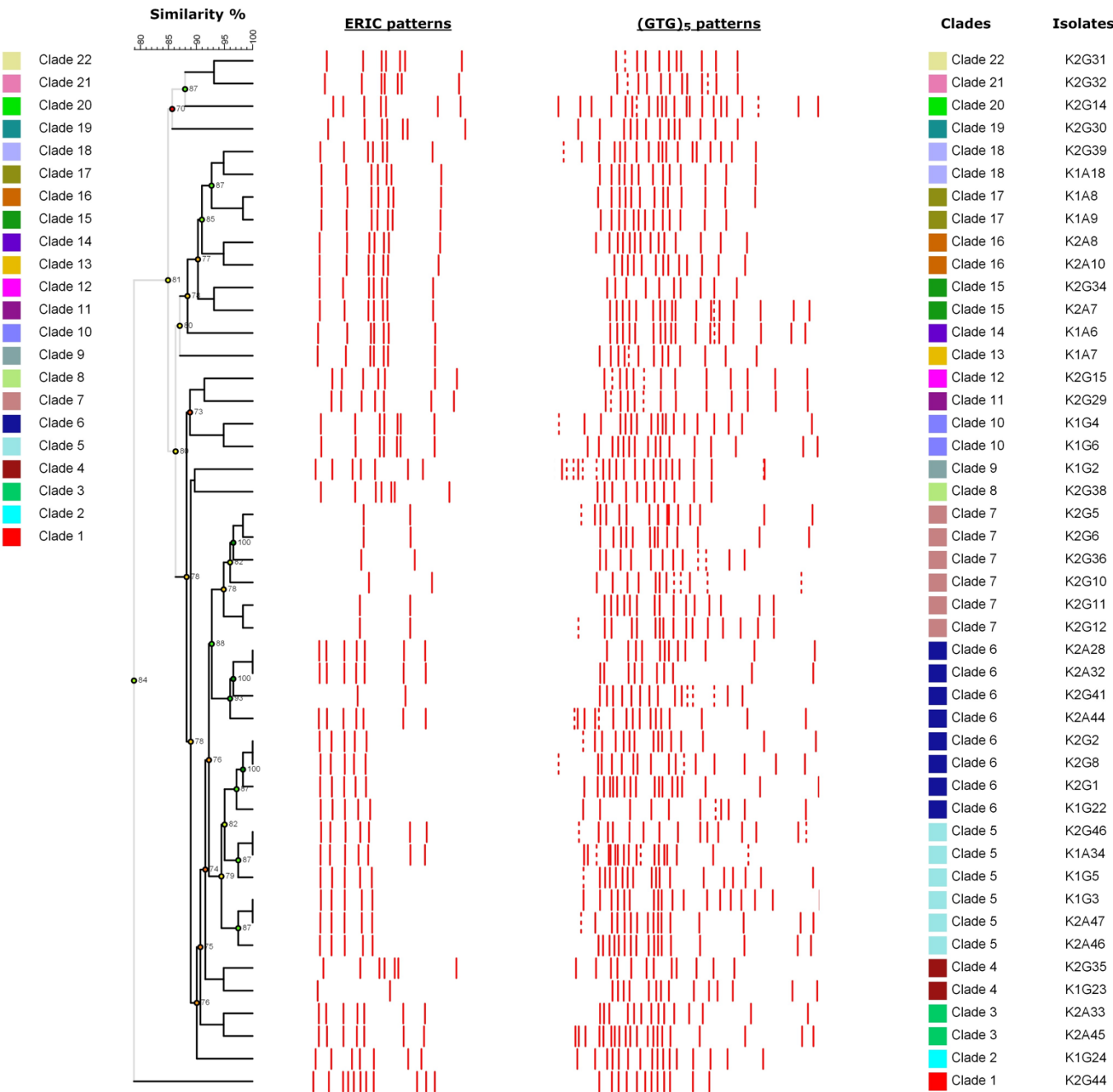
Figure 3. Dendrogram obtained combining the digitalized patterns from both (GTG)5 rep-PCR and ERIC rep-PCR. The dendrogram was drawn from UPGMA analysis using Dice's coefficient. The discrimination of biotypes was performed considering a similarity threshold of 94%. The cophenetic coefficient was represented by numbers and dots colored as red-orange-yellow-green, based on the branch quality.

4. Discussion

Bacterial cellulose is receiving great attention due to several features that make it suitable for many biotechnological applications, such as biomedical devices, food packaging components and engineering materials [1,2,30].

It is well known from the literature that Kombucha tea represents one of the most abundant reservoirs of BC producer bacteria, in particular strains belonging to the *Komagataeibacter* genus [8,10].

In this study, Kombucha tea was chosen as a selective source for recovering cellulose producing bacteria. 46 isolates were selected as the main component of the liquid and pellicle fractions of GTK and BTK Kombucha tea samples and their phenotypic and genotypic variability was evaluated. RFLP of 16S rRNA and an analysis of the interspecific variability of isolates was conducted and results discussed considering the available genome sequences of strains K1G4 and K2G30.

The data obtained by RFLP analysis showed a very low variability in terms of the number of different species isolated from Kombucha tea. This result is in agreement with previous studies reflecting both the low species diversity in Kombucha tea [8], as well as the difficulty of taxonomic assignment of *Acetobacteraceae* by using 16S rRNA gene, due to its similarity in high phylogenetic related organisms, as extensively reported in the literature [31].

Considering the analysis of the intraspecific variability within bacteria, usually, genotyping is performed using the amplification of highly conserved tandem repeat regions widely dispersed in the genome. The amplification of repeated elements of genomic DNA, such as $(GTG)_5$ and ERIC sequences, offers some advantages in terms of costs and rapidity, and are well developed for several gram-negative and gram-positive bacterial species, such as *Lactobacilli* [32] and *Enterococci* [33] and acetic acid bacteria [34].

In this study, the clustering analysis using the digitalized patterns of $(GTG)_5$ elements revealed the clustering of the 46 isolates into 5 large clusters considering a cluster cut-off value of 50% in similarity. The discrimination power of this analysis was 0.94 considering a similarity threshold of 70%. At this similarity value, a total of 20 different biotypes were discovered. Similar results were obtained using the amplification of ERIC repeated elements. Considering the cluster cut-off analysis, the comparison of the digitalized patterns of ERIC rep-PCR showed a clustering of the 46 isolates with a similarity percentage of 50%, the same result obtained using $(GTG)_5$ rep-PCR. The discrimination power of ERIC fingerprint was 0.95, very similar to the $(GTG)_5$ rep-PCR, considering a similarity percentage of 70. Based on these results, a total of 19 different clades were identified, compared to the 20 obtained using $(GTG)_5$ rep-PCR. In both the fingerprint approaches, K1G4 and K2G30, previously identified as *K. xylinus* [12,29], were considered as reference strains and they were clustered into two different clades. Among the two reference strains, the percentage of similarity was 44.2% in the case of $(GTG)_5$ rep-PCR and 56.7% for ERIC rep-PCR. These results support the hypothesis that most of the strains analysed belong to the *K. xylinus* species and that the discrimination was performed at the strain level.

In order to improve the genotyping analysis, a combined model of the two fingerprint assays was performed (Fig. 3). The resulting analysis showed a clusterization of the isolates into 7 clades based on the cluster cut-off value of 88% of pattern similarity. The discrimination power of this analysis was 0.94 at 94% of similarity. The number of biotypes defined by the combined model was 22, meaning an improvement of the discrimination of the isolates, compared to the single results obtained by the two approaches. Concerning the reference strains (K1G4 and K2G30) also in this case we observed a grouping into two different clades, with a similarity percentage of 84.9%, meaning that all the isolates with equal or major than that value possibly belong to the species of *K. xylinus*. Based on this consideration, all of the isolates could be assigned to the *K. xylinus* except one isolate, the K2G44, clustering out of the group, with a similarity percentage of 78.8%. The data were in agreement with previous studies of fingerprinting

analysis of acetic acid bacteria [34,35], in which the differences of strains among the same species (as *Komagataeibacter xylinus*) were about 80%. In this case, combining the two methods, the detection of different strains was improved.

The results obtained in this study reveal a high variability in producing BC by strains of the *Komagataeibacter* genus and define Kombucha tea as an optimal reservoir of cellulose producing bacteria.

Author Contributions

M.G. conceptualized the research, provided resources and supervised the work; D.M. performed experiments; S.L.C. performed genotyping analysis and wrote part of the original draft; L.D.V. reviewed and edited the manuscript; K.A. wrote part of the original draft; M.B .participated in the investigation, reviewed and edited the manuscript.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Robotti, F.; Bottan, S.; Fraschetti, F.; Mallone, A.; Pellegrini, G.; Lindenblatt, N.; Starck, C.; Falk, V.; Poulikakos, D.; Ferrari, A. A micron-scale surface topography design reducing cell adhesion to implanted materials. *Sci. Rep.* **2018**, doi:10.1038/s41598-018-29167-2.
2. Haghighi, H.; Gullo, M.; La China, S.; Pfeifer, F.; Siesler, H.W.; Licciardello, F.; Pulvirenti, A. Characterization of bio-nanocomposite films based on gelatin/polyvinyl alcohol blend reinforced with bacterial cellulose nanowhiskers for food packaging applications. *Food Hydrocoll.* **2020**, 106454, doi:10.1016/j.foodhyd.2020.106454.
3. Valera, M.J.; Torija, M.J.; Mas, A. Detrimental effects of acetic acid bacteria in foods. In *Acetic Acid Bacteria: Fundamentals and Food Applications*, 1st ed.; Sengun, I., Eds.; CRC Press, Taylor & Francis Group: Florida, USA, 2017; volume 10, pp. 299–320.
4. Gullo, M.; La China, S.; Falcone, P.M.; Giudici, P. Biotechnological production of cellulose by acetic acid bacteria: current state and perspectives. *Appl. Microbiol. Biotechnol.* **2018**, 1–14, doi:10.1007/s00253-018-9164-5.
5. Son, H.J.; Heo, M.S.; Kim, Y.G.; Lee, S.J. Optimization of fermentation conditions for the production of bacterial cellulose by a newly isolated *Acetobacter* sp. A9 in shaking cultures. *Biotechnol. Appl. Biochem.* **2001**, *33*, 1, doi:10.1042/ba20000065.
6. Coton, M.; Pawtowski, A.; Taminiau, B.; Burgaud, G.; Deniel, F.; Coulloumme-Labarthe, L.; Fall, A.; Daube, G.; Coton, E. Unraveling microbial ecology of industrial-scale Kombucha fermentations by metabarcoding and culture-based methods. *FEMS Microbiol. Ecol.* **2017**, *93*, doi:10.1093/femsec/fix048.
7. Laureys, D.; Britton, S.J.; De Clippeleer, J. Kombucha tea fermentation: a review. *J. Am. Soc. Brew. Chem.* 2020, *78*, 165–174.
8. Sievers, M.; Lanini, C.; Weber, A.; Schuler-Schmid, U.; Teuber. M. Microbiology and fermentation balance in a Kombucha beverage obtained from a tea fungus fermentation. *Syst Appl Microbiol.* **1995**, *18*, 590-594, doi:10.1016/S0723-2020(11)80420-0.
9. Arıkan, M.; Mitchell, A.L.; Finn, R.D.; Gürel, F. Microbial composition of Kombucha determined using amplicon sequencing and shotgun metagenomics. *J. Food Sci.* **2020**, *85*, 455–464, doi:10.1111/1750-3841.14992.
10. Villarreal-Soto, S.A.; Beaufort, S.; Bouajila, J.; Souchard, J.P.; Taillandier, P. Understanding Kombucha tea fermentation: a review. *J. Food Sci.* **2018**, *83*, 580–588, doi:10.1111/1750-3841.14068.
11. Gaggìa, F.; Baffoni, L.; Galiano, M.; Nielsen, D.; Jakobsen, R.; Castro-Mejía, J.; Bosi, S.; Truzzi, F.; Musumeci, F.; Dinelli, G.; et al. Kombucha beverage from green, black and rooibos teas: a comparative study looking at microbiology, chemistry and antioxidant activity. *Nutrients* **2018**, *11*, 1, doi:10.3390/nu11010001.
12. Gullo, M.; La China, S.; Petroni, G.; Di Gregorio, S.; Giudici, P. Exploring K2G30 genome: a high bacterial cellulose producing strain in glucose and mannitol based media. *Front. Microbiol.* **2019**, *10*, 1–12, doi:10.3389/fmicb.2019.00058.
13. Singhsa, P.; Narain, R.; Manuspiya, H. Physical structure variations of bacterial cellulose produced by different *Komagataeibacter xylinus* strains and carbon sources in static and agitated conditions. *Cellulose* **2018**, *25*, 1571–1581, doi:10.1007/s10570-018-1699-1.
14. Mamlouk, D.; Gullo, M. Acetic acid bacteria: physiology and carbon sources oxidation. *Indian J. Microbiol.* 2013, *53*, 377–384.
15. Jayabalan, R.; Marimuthu, S.; Swaminathan, K. Changes in content of organic acids and tea polyphenols during kombucha tea fermentation. *Food Chem.* **2007**, *102*, 392–398, doi:10.1016/j.foodchem.2006.05.032.
16. Mamlouk, D. Insight into physiology and functionality of acetic acid bacteria through a multiphasic approach. [Dissertation], University of Modena and Reggio Emilia. Reggio Emilia, Italy, **2012**.

17. De Vero, L.; Boniotti, M.B.; Budroni, M.; Buzzini, P.; Cassanelli, S.; Comunian, R.; Gullo, M.; Logrieco, A.F.; Mannazzu, I.; Musumeci, R.; et al. Preservation, characterization and exploitation of microbial biodiversity: the perspective of the Italian Network of Culture Collections. *Microorganisms* **2019**, *7*, 685, doi:10.3390/microorganisms7120685.

18. Gullo, M.; Mamlouk, D.; De Vero, L.; Giudici, P. *Acetobacter pasteurianus* strain AB0220: cultivability and phenotypic stability over 9 years of preservation. *Curr. Microbiol.* **2012**, *64*, 576–580, doi:10.1007/s00284-012-0112-9.

19. Navarro, R.; Uchimura, T.; Komagata, K. Taxonomic heterogeneity of strains comprising *Gluconacetobacter hansenii. J. Gen. Appl. Microbiol.* **1999**, *45*, 295–300, doi:10.2323/jgam.45.295.

20. Gullo, M.; Sola, A.; Zanichelli, G.; Montorsi, M.; Messori, M.; Giudici, P. Increased production of bacterial cellulose as starting point for scaled-up applications. *Appl. Microbiol. Biotechnol.* **2017**, *101*, 8115–8127, doi:10.1007/s00253-017-8539-3.

21. Hwang, J.W.; Yang, Y.K.; Hwang, J.K.; Pyun, Y.R.; Kim, Y.S. Effects of pH and dissolved oxygen on cellulose production by *Acetobacter xylinum* BRC5 in agitated culture. *J. Biosci. Bioeng.* **1999**, *88*, 183–188, doi:10.1016/S1389-1723(99)80199-6.

22. Gullo, M.; De Vero, L.; Giudici, P. Succession of selected strains of *Acetobacter pasteurianus* and other acetic acid bacteria in traditional balsamic vinegar. *Appl Env Microbiol.* **2009,** 75, 2585-2589, doi:10.1128/AEM.02249-08.

23. Versalovic, J.; Schneider, M.; Bruijn, F.J. De; Lupski, J.R. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods Mol. Cell. Biol.* **1994**, *5*, 25–40.

24. Versalovic, J.; Koeuth, T.; Lupski, R. Distribution of repetitive DNA sequences in *eubacteria* and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* **1991**, *19*, 6823–6831, doi:10.1093/nar/19.24.6823.

25. Hunter, P.R.; Gaston, M.A. Numerical index of the discriminatory ability of typing systems: An application of Simpson's index of diversity. *J. Clin. Microbiol.* **1988**, *26*, 2465–2466, doi:10.1128/jcm.26.11.2465-2466.1988.

26. Battikh, H.; Chaieb, K.; Bakhrouf, A.; Ammar, E. Antibacterial and antifungal activities of black and green kombucha teas. *J. Food Biochem.* **2013**, doi:10.1111/j.1745-4514.2011.00629.x.

27. Sievers, M.; Sellmer, S.; Teuber, M. *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in central Europe. *Syst. Appl. Microbiol.* **1992**, *15*, 386–392, doi:10.1016/S0723-2020(11)80212-2.

28. Ludwig W., Klenk H.P.; Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics. In *Bergey's manual of systematic bacteriology. The Proteobacteria, Part A, introductory essays*, 2nd ed.; Brenner, D.J., Krieg, N.R., Staley, J.T., Garrity, G.M. Eds.; Springer, New York, USA, (2005); Volume 2, pp. 49–65.

29. La China, S.; Bezzecchi, A.; Moya, F.; Petroni, G.; Di Gregorio, S.; Gullo, M. Genome sequencing and phylogenetic analysis of K1G4: a new *Komagataeibacter* strain producing bacterial cellulose from different carbon sources. *Biotechnol. Lett.* **2020**, *42*, 807–818, doi:10.1007/s10529-020-02811-6.

30. Jakmuangpak, S.; Prada, T.; Mongkolthanaruk, W.; Harnchana, V.; Pinitsoontorn, S. Engineering bacterial cellulose films by nanocomposite approach and surface modification for biocompatible triboelectric nanogenerator. *ACS Appl. Electron. Mater.* **2020**, *2*, 2498–2506, doi:10.1021/acsaelm.0c00421.

31. Cleenwerck, I.; Vandemeulebroecke, K.; Janssens, D.; Swings, J. Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae* sp. nov. and *Acetobacter malorum* sp. nov. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 1551–1558, doi:10.1099/00207713-52-5-1551.

32. Gevers, D.; Huys, G.; Swings, J. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol. Lett.* **2001**, *205*, 31–36, doi:10.1111/j.1574-6968.2001.tb10921.x.

33. Švec, P.; Vancanneyt, M.; Seman, M.; Snauwaert, C.; Lefebvre, K.; Sedláček, I.; Swings, J. Evaluation of (GTG)$_5$-PCR for identification of *Enterococcus* spp. *FEMS Microbiol. Lett.* **2005**, *247*, 59–63, doi:10.1016/j.femsle.2005.04.030.

34. De Vuyst, L.; Camu, N.; De Winter, T.; Vandemeulebroecke, K.; Van De Perre, V.; Vancanneyt, M.; De Vos, P.; Cleenwerck, I. Validation of the (GTG)$_5$-rep-PCR fingerprinting technique for rapid classification and identification of acetic acid bacteria, with a focus on isolates from Ghanaian fermented cocoa beans. **2007**, doi:10.1016/j.ijfoodmicro.2007.02.030.

35. Cleenwerck, I.; De Wachter, M.; González, Á.; De Vuyst, L.; De Vos, P. Differentiation of species of the family *Acetobacteraceae* by AFLP DNA fingerprinting: *Gluconacetobacter kombuchae* is a later heterotypic synonym of *Gluconacetobacter hansenii. Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 1771–1786, doi:10.1099/ijs.0.005157-0.