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ANTIMICROBIAL POTENTIAL OF KOMBUCHA BACTERIAL BIOPOLIMER

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Abstract

During the process of the tea fermentation with Kombucha microbial consortium (SCOBY), due to the presence of acetic bacteria (Acetobacter, Gluconacetobacter) a cellulosic biopolymer is developing on the surface. On industrial level, generally, this is considered as a residue, but recent research proved that such waste can be used for different purposes in pharmaceutical and cosmetic industry as excipient or bandage on injured skin. In this context, we have conducted Kombucha fermentation on different tea sources (black tea, green tea, oolong tea and green tea added with Melissa officinalis). The polymers obtained after fermentation processes were dried under controlled conditions and tested for its antimicrobial activity on potential pathogenic microorganisms (Gram-positive and Gram-negative bacteria, as well as on different Candida species). The highest quantity of polymer was obtained on Green tea supplemented with Melissa, followed by oolong tea, green tea and black tea. All polymers showed antibacterial activity on all Gram-positive and Gram-negative bacteria, including on methicillin resistant S. aureus; highest antibacterial activity was registered in the case of oolong tea source, followed by green tea, green tea with Mellissa and black tea. No inhibitory activity was noticed in the case of Candida species.

Key words*: antimicrobial activity, bacterial cellulose, Kombucha.*

INTRODUCTION

Nowadays, Kombucha has already received the status of functional drink (Jayabalan et al. 2019), accepted by many consumers, especially in Asia, as an alternative to the sugary beverages. During the process of the tea fermentation with Kombucha microbial consortium (SCOBY) a biofilm (cellulosic polymer) is formed on the surface (Figure 1). On industrial level this is considered as a residue, but recent research proved that such waste can be used for different purposes in pharmaceutical and cosmetic industry as excipient or bandage on injured skin. The biofilm is developed by a complex microbial consortium consisting of bacteria such as *Rhodospirillales* cluster which includes *Acetobacter, Bacteria xylinum* and *Gluconobacter*) (Dutta et al., 2007; Nguyen et al., 2008). One of the specific biochemical activity of this acetic bacteria is the oxidation of glucose to gluconic acid, which further leads to the synthesis of microbial cellulose forming the biofilm that remains in the liquid surface. This form of cellulose production has the advantage that, under controlled conditions, the bacteria grow rapidly and can produce cellulose from a variety of carbon sources including glucose, ethanol, sucrose, and glycerol (Villa-Real et al., 2018). The microbial cellulose is produced extracellularly in the form of fibrils that are attached to the bacterial cell. Bacteria produce two forms of cellulose, cellulose I and cellulose II. Cellulose I is a ribbon-like polymer composed of bundles of microfibrils, while cellulose II is an amorphous polymer that is thermodynamically more stable than cellulose I (Podolich et al., 2016). During the fermentation ongoing, the membrane thickness is increased by the generation of new layers on the surface, forming a suspended structure in the culture medium.

The SCOBY contains also other various microorganisms, like yeast belonging to *Pichia* spp., *Torulopsis* spp., *Zygosaccharomyces* spp. or *Brettanomyces* spp. (Greenwalt et al., 2000; Vina et al., 2004), as well as lactic bacteria like *Lactobacillus* sp., *Lactococcus* sp. or *Lecunosctoc* sp. (Marsh et al., 2014; Chakravorty et al., 2016).

The antimicrobial activity of Kombucha was not so extensively studied, more attention going toward its antioxidant and anti-carcinogenic effects. Data is provided only on the Kombucha soup (suspension), but no information was published, on our knowledge, in regard to the inhibitory activity of the biofilm. It is well known that the tea itself has been reported to have antimicrobial activity (Bansal et al., 2013). Many components such as organic acids (acetic acid, gluconic acid, glucuronic acid etc.), bacteriocins, proteins, enzymes, tea polyphenols and their derivatives which are produced during Kombucha fermentation have been said to contribute to its antimicrobial activity as well as other pharmacological effects (Bhattacharya et al., 2016). In early 2000, Sreeramulu and his teamproved that Kombucha exert antimicrobial activities against *S. typhimurium*, *E. coli*, *Sh. sonnei*, *C. jejuni* and *S. enteritidis* even at neutral pH and after thermal denaturation; their finding suggested the presence of antimicrobial compounds other than large proteins and acetic acid in Kombucha. Recently, the Kombucha suspension made of different tea sources was proven to have inhibitory activity on grape mould *Botrytis cinerea* (Matei B. et al., 2017). Also, Ansari et al. (2017) reported that the Kombucha green tea has an antibacterial activity against *Staphylococcus aureus* and *Salmonella typhimurium*.

One report on bacterial cellulose antimicrobial activity was provided by Kukharenko et al. (2014). After combining bacterial cellulose from *Gluconacetobacter xylinus* isolated from Kombucha with polymeric biocide polyhexamethylene guanidine hydrochloride (PHMG-Cl), the polymer showed excellent efficacy against multidrug resistant strains *Staphylococcus aureus* and *Klebsiella pneumonia,* as well as on yeast.

The aim of this work was to test the antimicrobial activity of Kombucha cellulosic polymers from four different tea sources, respectively black tea, green tea, oolong tea and green tea added with *Melissa officinalis*.

MATERIALS AND METHODS

Production of Kombucha biofilm

Kombucha samples were prepared adding in boiled water 6 g/l of different tea (*Camellia sinensis*) sources: black tea (KN), green tea (KV), oolong tea (KO) and green tea added with *Melissa officinalis* (KVM) from the market; the infusion time was 15 minutes. The sweetened tea (90 g sucrose/l), after cooling, was inoculated 5% Kombucha SCOBY suspension from former cultures maintained in the laboratory at room temperature, aside the direct action of sun light. The fermentation was conducted during 17 days at 28ºC. In the end of the fermentation, the upper polymer was harvested and dried under ventilation at 35ºC for 18 days; its weight was measured by an analytical balance.

Culture media

To test the antimicrobial activity specific media for each microbial group were used, as follows: Nutrient Agar (Merck, Germany) and Nutrient Broth (Merck, Germany) for bacterial use and YPG (Oxoid) and Malt Extract Agar (Roth, UK) for the fungi. All media were autoclaved at 121ºC for 15 minutes.

Tested microorganisms

The inhibitory tests were performed on thirteen potential pathogenic bacteria and on four *Candida* species (Table 1). For the inhibitory activity tests the bacterial inoculum was prepared in nutrient broth for 48 hours at 37ºC, while the fungi, in liquid YPG for 24 hours at 28ºC.

Table 1. Tested microorganisms for their susceptibility to be inhibited by Kombucha polymers

No.	Tested microorganisms	Origin
1.	*Bacillus cereus* CP1	Faculty of Biotechnology collection
2.	*Bacillus pumilus* MI 26	Faculty of Biotechnology collection
3.	*Bacillus licheniformis* MI 27	Faculty of Biotechnology collection
4.	*Staphylococcus aureus* ATCC 25923	American Type Culture Collection
5.	*S. aureus* ATCC 6538	American Type Culture Collection
6.	*S. aureus* ATCC 43300 MRSA	American Type Culture Collection
7.	*S. epidermidis* ATCC 51625 MRSE	American Type Culture Collection
8.	*S. epidermidis* ATCC 12228 MSSE	American Type Culture Collection
9.	*Enterococcus faecalis* MI 28	Faculty of Biotechnology collection
10.	*Salmonella typhimurium* ATCC 14028	American Type Culture Collection
11.	*Escherichia coli* ATCC 8739	American Type Culture Collection
12.	*Pseudomonas aeruginosa* ATCC 9027	American Type Culture Collection
13.	*Candida albicans* ATCC 10231	American Type Culture Collection
14.	*Candida parapsilosis* ATCC 20019	American Type Culture Collection
15.	*Candida guilermondii* MI 40	Faculty of Biotechnology collection
16.	*Candida krusei* MI 41	Faculty of Biotechnology collection

Inhibitory activity test

For the inhibitory activity was employed an adapted method of the disk diffusion test (Kirby Bauer test) in which the antibiotic discs were replaced with discs made of the Kombucha cellulosic polymer (5 mm diameter). As positive controls were used ampicillin disk of 10 µg (BioAnalyse, Turkey) in the case of bacteria and fluconazole of 25 µg (BioAnalyse, Turkey) for the fungi. The formed halo was measured; the plates were performed in duplicate.

RESULTS AND DISCUSSIONS

Production of Kombucha polymers

After 17 days of fermentation, the membrane developed on the upper phase of the suspension (Figure 1) was harvested and dried at 35ºC.

Figure 1. Aspects of Kombucha biofilm (polymer) developed after 17 fermentation days in black tea

Aspects of the dried biomass can be observed in Figure 2, while the microfiber developed by the acetic bacteria activity in black tea is presented in Figure 3 (under optical microscopy, 10x). In the end, the dried biomass was weight.

Figure 2. Aspects of Kombucha biofilms dried during 18 days at 35ºC (A: black tea; B: oolong tea; C: green tea; D: green tea + *Melissa officinalis*)

Under the same fermentation conditions, adding the same size inoculum in different tea types, the highest quantity of polymer biomass was obtained in the case of the green tea added with *Melissa officinalis* (KVM: 6.6 g/l Kombucha suspension), while the lowest quantity was registered for the black tea (KN: 3.5 g/l Kombucha suspension), being almost half of the KVM (Figure 4).

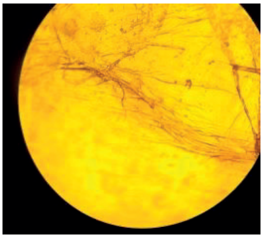

Figure 3. Optic microscopy image of Kombucha polymer microfibre made of black tea (KN) (10x)

In the case of oolong (KO) and green tea (KV) was obtained close quantities of biomass (5.3-5.5 g/l); oolong tea, also named blue-green tea, is a semi-fermented tea, with closer biochemical composition to the green tea, than the black tea, which is a completed fermented product (more oxidized); all are made of *Camellia sinensis* leafs under different processing conditions.

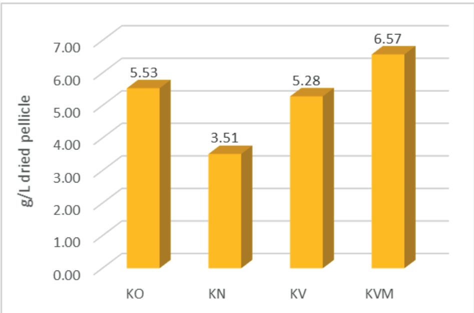

Figure 4. Dried Kombucha biofilm biomass obtained from different tea sources: black tea (KN); green tea (KV); oolong tea (KO); green tea + *Melissa officinalis* (KVM)

Antimicrobial activity of Kombucha polymers

Regarding the inhibitory activity on the Gram-positive bacteria (Table 2 and Figure 5), all Kombucha biofilms showed high inhibitory activity against the *E. faecalis*, higher than the antibiotic control, the ampicillin of 10 µg. Also, the addition of *Melissa officinalis* in the green tea led to a product with high inhibitory activity on *B. licheniformis*. Except the staphylococci, all biofilms exhibited moderate inhibitory activity on all Gram-positive bacteria. However, should be emphasized that all the biofilms still had some inhibitory activity on all methicilin resistant staphylococci, while the ampicillin, hasn't showed any inhibitory activity.

Table 2. Inhibitory activity of Kombucha biofilm on potential pathogenic Gram-positive and Gram-negative bacteria

Bacteria species	Amp. (10 µg)	KO	KN	KVM	KV
Gram-positive bacteria					
B. cereus CP1	-	++	++	++	++
B. licheniformis MI 27	++	++	++	+++	++
B. pumilus MI 26	+++	++	+	++	++
E. faecalis MI 28	++	+++	+++	+++	+++
S. aureus ATCC 25923	++	+	+	+	+
S. aureus ATCC 6538	+++	+	+	+	+
S. aureus ATCC 43300 MRSA	-	+	+	+	+
S. epidermidis ATCC 51625 MRSE	-	+	+	+	+
S. epidermidis ATCC12228 MSSE	-	+	+	+	+
Gram-negative bacteria					
S. typhimurium ATCC 14028	++	++	+	++	++
E. coli ATCC 8739	-	++	++	++	++
P. aeruginosa ATCC 9027	-	++	+	++	+

Legend: Amp (Ampicillin); (-): no inhibitory activity; (+): low inhibitory activity; (++): moderate inhibitory activity: (+++): high inhibitory activity

Also, the inhibitory activity was tested on Gram-negative bacteria with pathogenic potential (Table 2 and Figure 6). No high inhibitory activity (+++) was noticed against such potential pathogenic bacteria. However, most of the Kombucha biofilm inhibited moderately the development of Gram-negative bacteria, especially the *E. coli* strain.

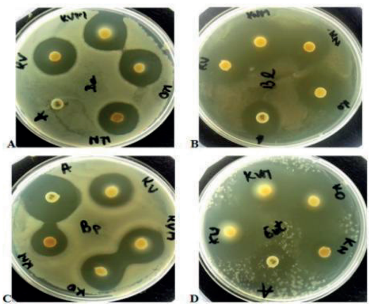

Figure 5. Inhibitory activity of Kombucha biofilm on different Gram-positive bacteria:
A - *B. cereus* CP1; B - *B. licheniformis* MI 27; C - *B. pumilus* MI 26; D - *E. faecalis* MI 28

As a general remark, the biofilm made of black tea showed less remarkable antibacterial activity, while the most promising is the green tea added with *Mellisa officinalis*. *Mellisa officinalis* it was previously reported having inhibitory activity in aqueous extract against *Listeria monocytogenes* and *S. aureus* (Purcaru et al., 2018). Some other plants were reported to increase the antimicrobial effect of Kombucha suspension. Battikh et al. (2013) reported that the antimicrobial activity observed in the fermented infusions with *Lippia citriodora*, *Rosmarinus officinalis*, *Foeniculum vulgare* and *Mentha piperita* was not only significant against the tested Gram-positive and Gram-negative pathogenic bacteria, but also against all *Candida* strains tested except *C. krusei*.

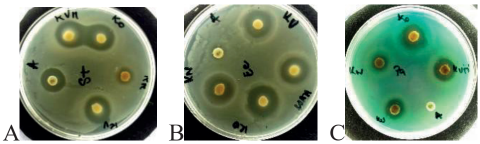

Figure 6. Inhibitory activity of Kombucha biofilm on different Gram-negative bacteria:
A - *S. typhimurium* ATCC 14028 (St); B - *E. coli* ATCC 8739; C - *P. aeruginosa* ATCC 9027

Another step was to test the action of Kombucha biofilms on some *Candida* spp. with potential pathogenic characteristics. None of the tested biomass show any inhibitory activity (Table 3), despite former results reported by Bathik et al (2013) on Kombucha suspension.

Table 3. Inhibitory activity of Kombucha biofilm on potential pathogenic *Candida* spp.

Candida spp.	Fluc. (25µg)	KO	KN	KVM	KV
C. albicans ATCC 10231	+++	-	-	-	-
*C. parapsilosis*ATCC20019	+++	-	-	-	-
*C. guilliermondii*MI 40	+++	-	-	-	-
*C. krusei*MI 41	+++	-	-	-	-

Legend: Fluc (Fluconazole); (-): no inhibitory activity; (+): low inhibitory activity; (++): moderate inhibitory activity: (+++): high inhibitory activity

In our study we have produced on lab scale bacterial biofilm starting from different tea infusion sources; we have obtained the lowest biofilm quantity in the case of the black tea, which is not in total accordance with data reported by El-Salam (2012). It may be presumed that some of the biochemical compounds of the black tea may inhibit the development of the bacteria group responsible for the biofilm development.

CONCLUSIONS

The use of different tea sources for Kombucha fermentation have an important influence on the quantity of the produced polymeric biofilm. In our trials, the highest dried biomass was obtained in the case of green tea added with *Melissa officinalis* (6.6 g/l Kombucha suspension), while the lowest quantity, almost half, was registered for the black tea (3.5 g/l Kombucha suspension.

Among the Gram-positive bacteria, the most inhibited specie was *Enterococcus faecalis* in the case of all the biofilms. An important finding is that, even the control, ampicillin, had not inhibitory activity on methicillin resistant staphylococci, still Kombucha biofilms have antagonistic effects, even on a low degree. In addition, most of the Kombucha biofilms inhibited moderately the development of Gram-negative bacteria, especially the *E.coli* specie. No inhibition was noticed against any *Candida* species taken into account in this work.

Our findings are coming to support the potential use of Kombucha bacterial biopolymer for the production of different pharmaceutical/medical devices, like bandages or cosmetic products, like masks, with antibacterial activity.

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