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# Bacterial Cellulose Powder from Tropical Fruits Byproducts: Characterization and Application in Smoothies

Running title: Smoothies with Bacterial Cellulose Powder from Tropical Fruit Byproducts

Rafael Sousa Cruz[1], Giovana Matias do Prado[1], Paulo Henrique Machado de Sousa[1], Nágila Maria Pontes Silva Ricardo[1], Débora Hellen Almeida de Brito[1], Francisco Ernani Alves Magalhães[2], Jéssica Azevedo Furtado[1] and Larissa Morais Ribeiro da Silva[1*]

[1]Federal University of Ceará, Campus do Pici, Ac. Público, 856 - Pici, 60020-181 Fortaleza, CE, Brazil
[2]Laboratory of Bioprospection of Natural Products and Biotechnology (LBPNB), 63660-000, Rua Solon Medeiros, S/N, BR 020, Bairro Bezerra e Sousa, Tauá, Ceará, Brazil

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## SUMMARY

*Research background.* The development of new products based on bacterial cellulose powder derived from tropical fruit byproducts (pulps and peels) represents a technological alternative that offers environmental benefits to everyone. This solution can be applied in both industrial and domestic settings. In this research, bacterial cellulose was produced from the fermentation of industrial waste from tropical fruits.

*Experimental approach.* Bacterial cellulose powders were produced via kombucha fermentation using agro-industrial byproducts from tropical fruits. The powders were selected and characterized according to physicochemical parameters, proximate composition, bacterial count, FTIR, TGA, and *in vivo* toxicity.

*Corresponding author:
Phone: +558533669744
E-mail: larissamrs@ufc.br

*Results and conclusions.* The powders presented pH values ranging from 4.5 to 2.49. The acerola bacterial cellulose showed the highest yield (6.25 %) and the highest vitamin C content ((1998.04±50.75) mg/100 g). Pseudoplastic behavior was observed in all smoothies, and the formulation containing bacterial cellulose from passion fruit showed higher viscosity. Zebrafish tests did not indicate any adverse effects related to the formulations.

*Novelty and scientific contribution.* The use of bacterial cellulose powders from agro-industrial waste appears as a healthy and sustainable alternative for the development of new products with a high content of vitamin C (acerola bacterial cellulose powder) or more viscous products (passion fruit bacterial cellulose powder).

**Keywords**: kombucha; co-product; powder; bacterial cellulose; industrial reuse

## INTRODUCTION

Kombucha is widespread in the East and West due to its popularity and its association with various therapeutic benefits (*1*). It is a beverage fermented from *Camelia sinenses* tea leaves by bacteria and yeast, which produces bacterial cellulose – a cellulosic film known as the Symbiotic Culture of Bacteria and Yeasts (SCOBY). This film forms on the surface of the liquid during fermentation (*2*) and consists of a range of microorganisms belonging to different genera, such as *Gluconobacter*, *Acetobacter*, *Zygosaccharomyces,* and *Saccharomyces* (*3*).

During fermentation, yeasts consume sucrose, hydrolyzing it into glucose and fructose, which are subsequently converted into ethanol and carbon dioxide. Meanwhile, bacteria produce gluconic acid and acetic acid. A wide variety of compounds are present in this beverage, including water-soluble vitamins, amino acids, pigments, lipids, proteins, hydrolytic enzymes, ethanol, polyphenols, minerals, and metabolic products of yeast and bacteria (*1,4*). Many of these compounds remain embedded in the bacterial cellulose, for which alternative uses must be considered as the beverage industry grows.

In recent years, several applications for this bacterial cellulose have been investigated, demonstrating significant potential for future use (*5*). For example, kombucha bacterial cellulose was used to create a new beer with acidic characteristics and high antioxidant activity (*6*). Additionally, nanoparticles were produced using yeast isolated from kombucha bacterial cellulose (*7*).

The growing consumption of fermented products worldwide has encouraged the food industry to explore alternatives that diversify these products, offering consumers new food options (*8-10*) and consequently generating waste from these processes.

Since fermentation is based on sugar consumption through the symbiotic association of

bacteria and yeast, it is possible to envision kombucha being prepared in different substrates beyond traditional tea (*3*).

Smoothies are an effective way to promote fruit and vegetable consumption. These beverages are considered rich sources of bioactive compounds and provide numerous health benefits (*11*).

Although smoothie consumption is already associated with various health benefits, numerous studies have focused on increasing their bioactive compound content to produce products with greater functional value. For instance, a strawberry and apple smoothie was developed by adding plant extracts, resulting in higher antioxidant activity (*12*). Similarly, a smoothie made from persimmon puree and apple was enhanced with plant extracts (*13*), and a strawberry smoothie with pomegranate extract was evaluated for its biological activities (*14*).

In this research, waste from the tropical fruit processing industry was used as a carbon source to produce bacterial cellulose through kombucha fermentation. Its potential as a new natural additive was evaluated to enhance viscosity and improve the nutritional value of smoothie-type beverages.

The selection of fruit processing waste was based on market availability – specifically, the pulps most consumed by the population, which consequently generate the largest accumulation of organic matter. This approach enables the production of other foods, thereby reducing environmental impact and organic waste (*15,16*).

The development of new foods, such as smoothies enriched with bacterial cellulose derived from tropical fruit waste, is a technological alternative that provides environmental benefits and can be applied in both industrial and artisanal settings. With this in mind, the selection of raw materials for fermentation was based on the most commercially relevant fruits, which generate the greatest amount of industrial waste. Furthermore, sustainable food use reduces the production of organic waste. It can be associated with the generation of new foods, making it a promising alternative across different areas of the food industry.

This study aimed to obtain bacterial cellulose from the fermentation of tropical fruit residues. The bacterial cellulose produced was characterized and applied to a smoothie-type product.

## MATERIALS AND METHODS

### *Raw material*

Tropical fruit waste of acerola (*Malpighia emarginata*), araçá (*Psidium cattleianum*), pineapple (*Ananas comosus*), guava (*Psidium guajava*), mango (*Mangifera indica*), and passion fruit (*Passiflora edulis*) was made available by an industry (Nossa Fruta Brasil, Eusébio, CE/Brazil), from the 2021/2022 harvest, and collected in plastic packaging shortly after processing the pulps.

Green tea (Dr. Oetker®, Brazil), as well as the other ingredients used to prepare the smoothie,

namely sugar (União®, São Paulo, Brazil) and milk (Betânia, Fortaleza, Ceará, Brazil), were purchased at local market stores in the city of Fortaleza/CE.

The samples were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SISGEN) under accession number AA72205 through the Federal University of Ceará, Fortaleza, Brazil.

The initial cellulosic film used in this study was donated by our research group in Fortaleza - CE, and it is not possible to identify the production characteristics of the “mother of kombucha”. The bacterial cellulose obtained was kept refrigerated in the kombucha itself in the glass container until use. During the experiment, the bacterial cellulose was divided into subparts to compose each container with the aforementioned extracts of tropical fruit co-products, and each container was submitted to a new fermentation medium for 7 days before use.

*Use of fruit residues as an alternative substrate in the fermentation of kombucha*

Preliminary tests were carried out with different fruit byproducts (Fig. S1) to evaluate fermentation and the potential use of these byproducts for fermentation and for the production of bacterial cellulose. For the fermentation process, both the Symbiotic Culture of Bacteria and Yeasts (SCOBY) and the liquid from the end of the kombucha fermentation test (prepared beforehand) were used as the starter culture (provided by our research group in Fortaleza, Brazil). It is important to note that using cultures from traditional fermented products such as kombucha and kefir may yield different results.

The preparation of the formulations starts with the infusion phase (at (90±2) °C for 5 min) using drinking water, the specific fruit byproducts (13 % *m/V*), and 7 % sugar. After the infusion phase, the samples were filtered through felt tissue to remove solid residues. Then the resulting liquid was cooled ((24±2) °C) and $\varphi$(kombucha)=10 % and 20 % (*m/V*) bacterial cellulose (SCOBY) were added, starting the fermentation process in the presence of oxygen. The bacterial cellulose used for all formulations was from the same initial fermentation, and the same amount of this culture was used for all formulations. A kombucha was also prepared using green tea as a control. The fermentation process was carried out for 7 days at 35 ºC (data not shown).

After fermentation of the formulations, cellulose formation was observed (Fig. S2), and the dehydration stage was then followed to obtain the powder yield.

*Obtaining powders from selected bacterial celluloses*

For the bacterial cellulose freeze-drying process, the methodology applied followed that described by Nunes *et al.* (*17*), in which the samples were frozen and freeze-dried (Liobras, São

Paulo, Brasil) for 48 h at -50 °C. The freeze-dried material was crushed in a mortar using liquid nitrogen ($N_2$), as the freeze-drying process was inefficient due to the high sugar concentration in the cellulose. To preserve the material, low-density polyethylene packaging bags wrapped in aluminum foil were used and placed in a desiccator until analysis.

*Characterization of kombucha bacterial cellulose powders*

The powders obtained were subjected to physicochemical analyses (humidity, pH, soluble solids, total titratable acidity, vitamin C content, water activity, FTIR, and TGA), microbiological, and toxicological analyses.

*Physicochemical and yield analyses*

When obtaining the powdered material, the yield was determined using Equation 1 described by (*18*), with modifications:

$$Y=(F/S)\cdot 100 \quad /1/$$

where $Y$ is the yield (%), F is the dry mass ($m$), and S is the mass of the byproduct before drying (g).

Humidity was determined based on the moisture loss of samples dried in an oven (SolidSteel Ltda., Piracicaba - SP, Brasil) at 105 °C until constant mass (*19*).

The pH was determined potentiometrically with a digital pH meter (model 3505; Jenway, UK) calibrated with pH buffer solutions at 4.0 and 7.0 (*19*).

Soluble solids were measured using a portable digital refractometer (ASKO, São Leopoldo, Brazil), model RT 32, and the results expressed in °Brix (*19*).

Total titratable acidity was determined by potentiometric titration with 0.1 M NaOH under stirring until pH reached 8.1, and the results were expressed as % malic acid (*19*).

Ascorbic acid (vitamin C) content was determined by titration with a DFI solution (0.02 % 2,6-dichlorophenolindophenol) until a permanent light pink color, using 5 g of the sample diluted in 50 mL of oxalic acid (0.5 %), according to Strohecker and Henning (*20*). The results were expressed in mg/100 g of ascorbic acid (*19*).

Water activity ($a_w$) was determined by direct reading with a measuring device from the Aqualab instrument (AquaLabLITE, Decagon, Pullman, WA, USA), using a control sample of activated carbon as a blank (*19*).

*Fourier-transform infrared spectroscopy analysis*

Fourier transform infrared spectroscopy (FTIR) analysis was performed using an IRTracer-100 (Shimadzu, Japan) spectrometer in the infrared range between 4000 and 400 $cm^{-1}$ with a resolution

of 4 $cm^{-1}$ at room temperature on a KBr pellet, with 64 scans per sample to verify similarities in the composition among them, which followed the methodology described by Fontes et al. (*21*). The samples (in triplicate) were divided into two groups according to the pre-established formulations based on the mass yields of bacterial cellulose.

A range of 400 to 450 $cm^{-1}$ (mid-infrared region) was established with a resolution of 4 $cm^{-1}$ at room temperature, and the infrared spectra were submitted to multivariate analysis to assess similarity among the different samples (*21*).

*Thermogravimetric analysis*

Thermogravimetric analysis (TGA) was performed to verify the thermal stability of the components, highlighting mass losses in different temperature ranges. TGA curves were obtained with a TGA Q50 (TA Instruments, United States) at a heating rate of 10 °C/min over a temperature range of 25 to 900 °C, with an air flow rate of 60 mL/min and an initial sample mass of approx. 10 mg.

*Microbiological analyses*

Microbiological analyses were performed using classical plate count methods with serial dilutions, employing peptone water (Kasvi®, Curitiba, Brazil) as the diluent. Samples were inoculated using the surface plating technique for the enumeration of total mesophilic aerobic bacteria, yeasts, and acetic acid bacteria, using plate count agar (PCA; Kasvi®, Curitiba, Brazil), Sabouraud dextrose agar with chloramphenicol (SDA; Kasvi®, Curitiba, Brazil), and glucose yeast extract calcium carbonate (GYC; HiMedia®, Mumbai, India) medium, with incubation at 35 °C for 48 h, 22 °C for 5 days, and 30 °C for 72 h, respectively. For enumeration of lactic acid bacteria, the pour plate technique was used with de Man, Rogosa, and Sharpe (MRS) agar (Kasvi®, Curitiba, Brazil), incubated at 32 °C for 72 h. Results were expressed as colony-forming units per gram (CFU/g). Total coliforms, thermotolerant coliforms, and *Escherichia coli* counts were determined by the Most Probable Number (MPN) method, using lactose broth (LB; HiMedia®, Mumbai, India) incubated at 35 °C for 48 h for the presumptive test, and 2 % Brilliant Green bile broth (BGB; HiMedia®, Mumbai, India) and *E. coli* broth (EC; HiMedia®, Mumbai, India) for confirmation of total and thermotolerant coliforms, incubated at 35 °C for 48 h and 45.5 °C for 24 h, respectively. Growth with gas production was considered as confirmation of the presence of total coliforms and thermotolerant coliforms (*22-24*).

*Assessment of acute and locomotion toxicity of bacterial cellulose powders using zebrafish as an in vivo model*

To assess toxicity, tests were carried out on zebrafish (*Danio rerio*) using the methodology

proposed by Magalhães *et al.* (*25*). To carry out the tests, adult wild zebrafish (ZF) of both sexes were used, aged 60 to 90 days, measuring (3.5±0.5) cm and weighing (0.4±0.1) g. The fish were obtained from Agroquímica: Comércio de Produtos Veterinários LTDA, a supplier in Fortaleza (Ceará, Brazil). After obtaining the *in vivo* models, groups of 50 fish were acclimatized for 24 h in glass aquariums (40 cm×20 cm×25 cm) containing dechlorinated water (ProtecPlus® antichlorine) and air pumps with submerged filters, at 25 ºC and pH=7.0, with a 14:10 h light/dark circadian cycle. The fish received food (Spirulina®) *ad libitum* 24 h before the experiments.

The open-field test was carried out to evaluate changes, or the lack of them, in the motor coordination of fish, whether due to sedation and/or muscle relaxation (*26*). The animals (*N*=6 per group) were randomly selected, transferred to a damp sponge, and orally treated with 20 µL of bacterial cellulose powder (vehicle group) (*27*). A group of animals without treatment, called the naïve group, was included. After treatments, the animals were placed individually in glass beakers (250 mL) containing 150 mL of aquarium water to rest. After 1 h, the animals were added to glass Petri dishes (10 cm×15 cm) containing the same water as the aquarium, each marked with four quadrants to analyze locomotor activity by counting line crossings (CL). Using the CL value of the naïve group as a baseline (100 %), the percentage of locomotor activity (AL/%) was calculated individually during 0–5 min).

The acute toxicity study was conducted with adult zebrafish (*D. rerio*) according to the methodologies proposed by the Organization for Economic Co-operation and Development (*28,29*). The animals (*N*=6 per group) were treated with the same concentrations of powders used in the open field test, but the fish were left to rest for 96 h to analyze mortality. The vehicle group (sterile distilled water) served as the control. After 96 h, the number of dead fish in each group was recorded to determine the lethal concentration that killed 50 % of the animals (LC50), using the Trimmed Spearman-Karber method with a 95 % confidence interval (*30*).

*Application of powders in the smoothie*

After homogenizing the ingredients using a home blender (300 W, Arno, Brazil), the drink was packaged in 1000 mL PVC bottles, sealed, and stored under refrigeration ((6±2) °C) until analysis.

The beverage formulations were defined through preliminary tests. The bacterial cellulose powders used were selected based on yield assessment after the fermentation period. Acerola, passion fruit, and green tea were chosen; the results of the preliminary tests that led to this choice are presented below.

The experiment was conducted using four smoothie formulations, obtaining, at the end of the process, a beverage without the addition of bacterial cellulose powder (F0), and with the addition of

bacterial cellulose powder from acerola (F1), passion fruit (F2), and green tea (F3) kombucha, each produced in triplicate (Fig. S3), which were later subjected to chemical and microbiological analyses. Smoothies were prepared by mixing milk (30 %), frozen fruit pulp (30 % strawberry and 20 % banana), and bacterial cellulose powder (20 %). Homogenization was performed using a domestic blender (300 W, Arno, Brazil). After homogenizing the ingredients, each beverage was packaged in 1000 mL PVC bottles, sealed, and stored under refrigeration ((6±2) °C) until analysis. The preparation of the smoothie formulations followed the basic requirements of Good Manufacturing Practices.

*Smoothie characterization*

The smoothie formulations were analyzed for proximate composition.

The protein content was measured by determining total nitrogen (*19*) using the Kjeldahl's digestion method (Tecnal, Piracicaba-SP, Brazil). The main steps were digestion, distillation, and titration.

For the lipid content, the Soxhlet method (*19*) was used, which is based on a process of continuous extraction of lipids with the organic solvent hexane Dinâmica® (Indaiatuba-SP, Brasil) using a lipid Soxhlet extractor (Tecnal, Piracicaba-SP, Brazil), followed by the removal of this solvent by distillation, drying of the material in an oven (SolidSteel Ltda., Piracicaba - SP, Brasil) until constant mass and weighing of the residual material obtained.

The ash content (*19*) was determined by weighing the material after heating in a muffle furnace (SolidSteel Ltda., Piracicaba-SP, Brasil) at 550–570 °C until constant mass was achieved.

The carbohydrate content was estimated by difference, based on the determined values of the other constituents (moisture, ash, lipids, and proteins).

The rheological behavior of the smoothies was determined using a Brookfield Searle-type rotational rheometer with concentric cylinders, model R/S plus SST 2000 (Brookfield, MA, United States). The DG-DIN sensor was used. Rheological analyses were obtained by varying the strain rate from 108 to 500 $s^{-1}$ (ascending curve) and from 500 to 100 $s^{-1}$ (descending curve), with a time of 1 minute, and a reading of 25 points for each curve. Readings were taken in triplicate, and a new sample was used for each measurement (*31*).

*Statistical analysis*

The results of the physicochemical analyses were expressed as a mean and standard deviation, subjected to analysis of variance and Tukey's test at a 5 % level of significance using the Statistica® v. 7 program (*32*) with a significance level of 5 % ($p \leq 0.05$), and Excel (*33*) to tabulate the obtained data.

## RESULTS AND DISCUSSION

*Preliminary tests to evaluate the production of bacterial cellulose using fruit byproducts*

The substrates that showed the best performance in cellulose formation were passion fruit, acerola, and traditional green tea, with araçá, guava, pineapple, and mango substrates having the lowest masses (data not shown). The three best substrates, based on cellulose mass produced, were selected for characterization and application in smoothies.

The cellulose membranes, at the end of fermentation, presented different wet masses: araçá (57 g), pineapple (39.50 g), acerola (66.70 g), guava (41.22 g), mango (25.40 g), passion fruit (81.90 g), and green tea (60.80 g). The yeasts and bacteria inoculated into the beverage for fermentation are responsible for the growth of what is known as tea fungus, or bacterial cellulose. Acetic bacteria produced a cellulose network as a secondary fermentation metabolite, giving rise to a structure that resembles a mushroom (*3*). Initially, cellulose-producing microorganisms increased in population and consumed dissolved oxygen. With an increase in the population of microorganisms, cellulose production increases on the container surface. Thus, as time progresses, the membrane thickness increases as new layers form on its surface, forming suspended structures.

In most formulations tested, an increase in fermentation time was associated with a reduction in soluble solid content (Table 1) and a decrease in pH (increase in acidity), as is common in most fermentation processes.

For the control sample (fermented with green tea), this behavior was not observed for the soluble solids content. However, a reduction in pH was observed, indicating that the fermentation process occurred. The final pH values for the formulations ranged from 2.49 to 3.0.

After 24 h of fermentation, we observed the formation of films on the surfaces of some containers. According to Goh *et al.* (*34*), these variations in bacterial cellulose production depend greatly on the strains used, fermentation time, and the chemical compounds present in the fermentation medium.

At the end of the fermentation period (7 days), cellulose production was evaluated by measuring the mass of bacterial cellulose produced. The biofilms were removed from the containers and cleaned with distilled water to remove impurities.

After washing the membranes and removing excess water, they were sent to the dehydration stage, followed by characterization and application in the smoothie.

Acerola bacterial cellulose powder showed the best yield (6.25 %), followed by passion fruit bacterial cellulose powder (3.84 %), and green tea bacterial cellulose powder (3.95 %). The acerola bacterial cellulose yield showcased better productivity in terms of mass. This may be associated with the composition of the acerola residue, which is rich in dietary fibers, including cellulose,

hemicellulose, and pectin, and, due to this characteristic, may have contributed to better bacterial cellulose yields, as all samples were subjected to the same incubation conditions for fermentation.

*Influence of different fruit byproducts on the physicochemical characteristics of bacterial cellulose powder*

The acidity values of bacterial cellulose powders after fermentation varied between 3.19 mmol/L (for the bacterial cellulose powder with acerola), 3.77 mmol/L (for the passion fruit bacterial cellulose powder), and 4.15 mmol/L (for the green tea bacterial cellulose powder), as shown in Table 2. Food pH is considered an indicator of food safety. A pH within the acidic range, below 4.5, inhibits the growth of the main microorganisms responsible for foodborne diseases (*35*).

When comparing the results obtained from the powders with the normative instruction for kombucha (*36*), it is possible to observe that the acidity and pH analyses are in agreement with the findings in this research: in a range of 3.19 to 4.15 for acidity ($p>0.05$), and 2.77 to 2.90 for pH, to the acerola, passion fruit, and green tea bacterial cellulose's powders.

Regarding the vitamin C content, the highest was observed in the acerola bacterial cellulose powder ((1.998.04±50.75) mg/100 g), followed by the green tea bacterial cellulose powder ((393.46±4.17) mg/100 g), and finally the passion fruit bacterial cellulose powder ((163.29±3.09) mg/100 g). Vitamin C is an essential nutrient that plays an important role in the human body. Its presence in kombucha, especially when derived from acerola residue, can provide additional benefits to the beverage and contribute to its classification as a functional beverage, while also helping preserve the product through its antioxidant role. Furthermore, the presence of vitamin C may be a microbial growth factor to consider for bacterial cellulose mass yield. The consumption of foods rich in vitamin C has been growing, as it is directly associated with delaying cellular aging and reducing the incidence of degenerative diseases, cardiovascular diseases, inflammation, brain dysfunction, and other conditions. The recommended value for vitamin C is 45 mg/day (*37*). It is noteworthy that the vitamin C content present in the acerola bacterial cellulose powder was over 5x greater than the vitamin C content present in bacterial cellulose powder from green tea, demonstrating its potential use in the development of new foods with higher content of this vitamin.

Soluble solids presented levels of (6.67±0.32) $^{o}$Brix for the acerola bacterial cellulose powder, (6.13±0.06) $^{o}$Brix for the passion fruit bacterial cellulose powder, and (8.13±0.06) $^{o}$Brix for the green tea bacterial cellulose powder. During fermentation, soluble solids may decrease as sugars are consumed by bacteria and yeast in the bacterial cellulose. This measurement can be useful for controlling the sugar and nutrient content of the final beverage (*38*).

Studies show that new raw materials can serve as alternative substrates for the fermentation

of beverages similar to kombucha. In the research developed by Câmara *et al.* (*39*), using as an alternative means to obtain the kombucha beverage the residues of red guava, pineapple, cashew, mango, and mombim, and also through the analysis of the proximate composition, the authors highlighted the high nutritional value of the raw materials used, which influenced the preparation of kombuchas.

The protein contents of the green tea, passion fruit, and acerola bacterial cellulose powder samples were below 2 %, with the latter two showing higher protein contents (1.39 and 1.36 %, respectively). The findings of the research carried out by Moraes *et al.* (*40*) reported a protein value of 0.20 g/100 mL in the passion fruit kombucha sample, which is lower than the result obtained in this research.

According to the Brazilian Food Composition Table (*41*), 100 g of passion fruit contains 0.8 g of protein, a value added to its bacterial cellulose powder, and 100 g of acerola includes 0.4 g of protein.

The samples showed low lipid levels (0.23–0.60). This may be related to the fact that the fruits and green tea used are low in lipids (*41*). Bacterial cellulose powders generated with the addition of fruit co-products would contribute only around 0.66 % to the lipid intake.

The relative ash values obtained in this study were 1.08 to 2.58 %. The values obtained for ash are lower than those reported in studies by (*42*), which found values between 4.59 and 7 %.

Water activity ($a_w$) is an important factor in evaluating food stability, as it corresponds to the thermodynamically available water for chemical and biochemical reactions (*43*). Furthermore, aw can provide important data on the moisture content of raw materials.

The FTIR spectra (Fig. 1) showed similarities among the three samples and were consistent with the chemical structure identified in bacterial cellulose derived from acerola byproduct, as reported by (*44*). The analysis revealed characteristic bands associated with bacterial cellulose, including an OH-band at approximately 3369 $cm^{-1}$ and a CH stretch of $CH_2$ and $CH_3$ groups around 2935 $cm^{-1}$. Bands in the proximity of 1639 $cm^{-1}$ and 1423 $cm^{-1}$ were attributed to the glucose carbonyl group (C=O), as well as $CH_2$ bending and C–OH in-plane bending. Moreover, the spectral region between 1338 and 1240 $cm^{-1}$ indicated the presence of crystalline regions within the cellulose structure.

Carbohydrates, such as cellulose and other polysaccharides, have characteristic bands around 1000-1200 $cm^{-1}$ (C-O-C bond region) and 3000-3600 $cm^{-1}$ (O-H bond region). Proteins present in bacterial cellulose can exhibit characteristic bands around 1650-1700 $cm^{-1}$ (region of C=O peptide bonds) and 3100-3500 $cm^{-1}$ (region of N-H and O-H vibrations). Lipids can present bands in different areas, depending on their specific composition. Generally, absorption bands can be observed around 2800-3000 $cm^{-1}$ (C-H bond region) and 1700-1750 $cm^{-1}$ (C=O bond region of fatty acids). Polyphenols,

such as phenolic acids and flavonoids, can exhibit characteristic bands around 1600-1700 $cm^{-1}$ (region of aromatic C=C bonds) and 3200-3600 $cm^{-1}$ (region of O-H vibrations).

It is worth noting that the exact wavelengths of the bands may vary depending on the specific composition of the bacterial cellulose. In the particular case of passion fruit and green tea bacterial cellulose powders, the presence of functional compounds may vary depending on the unique chemical composition of the passion fruit and interactions with the bacterial cellulose.

These are just a few general differences in the functional compounds found in different bacterial cellulose powders. It is noteworthy that the specific composition may vary depending on the preparation method, variety of fruit or tea used, and other factors.

*Results of thermogravimetric analysis*

The thermogravimetric analysis (TGA) curves of acerola (A), passion fruit (B), and green tea (C) bacterial cellulose are shown in Fig. 2. All samples showed a similar decomposition behavior.

The first event is associated with sample dehydration, followed by the degradation of low-molecular-mass components in the sample, probably originating from the fermentation process. The event with initial degradation temperature ($T_{onset}$) at 254 °C (A), 219 °C (B), and 245 °C (C) presented maximum degradation rate ($T_{max}$) temperatures at 339 °C (A), 331 °C (B), and 321 °C (C), corresponding to the depolymerization and decomposition of glycosyl units. The total mass loss during the analysis was 81% (A), 79% (B), and 77% (C). The same behavior was previously reported by (*45*).

*Influence of different fruit byproducts on the microbiological characteristics of bacterial cellulose powder*

The results of the microbiological analyses (data not shown) indicate that the developed powders are safe for consumption from a microbial perspective, as they showed *E. coli* counts <3 MPN/mL.

For the lactic acid bacteria count, the results were $8.0 \cdot 10^2$ CFU/g for sample A, $3.0 \cdot 10^2$ CFU/g for sample B, and $4.0 \cdot 10^2$ CFU/g for sample C, results similar to those reported in studies by Binda and Ouwehand (*46*).

For the count of acetic acid bacteria, results of <10 CFU/g were obtained for samples A, B, and C. These bacteria have been associated with several health benefits (*47*). They also play a crucial role in kombucha production, as they produce acetic acid, which gives kombucha its characteristic flavor and contributes to its preservation.

The yeast count was <10 CFU/g for samples A, B, and C. It is important to note that different

yeasts may be present in bacterial cellulose, contributing to the flavor, aroma, and characteristics of kombucha. Five yeasts were found in the kombucha beverage produced from telang flower (*Clitoria ternatea* L.) tea (*48*). No data were found in the literature on the counting of these microorganisms in powdered bacterial cellulose.

*In vivo toxicity of bacterial cellulose powders*

In Fig. 3, the results of the open field test for bacterial cellulose powders of acerola (A), passion fruit (B), and green tea (C) are shown. The oral administration of powders diluted in water to animals did not cause motor impairment. This suggests that, under the experimental conditions used, ingestion of kombucha bacterial cellulose powders did not affect the animals' locomotor activity. Although there was a reduction in line crossings in the petri dish among animals treated with bacterial cellulose powders compared to the control and naïve groups, this difference was not statistically significant.

Regarding the acute toxicity test, no mortality was recorded for acerola, passion fruit, or green tea bacterial cellulose powders during the analyzed period (data not shown). Therefore, powders developed with alternative substrates (acerola and passion fruit) appear to be safe for human consumption, without presenting toxicity capable of compromising human health.

From the results obtained, the concentrations tested for the three samples are safe, with CL50 values (lethal concentration for 50 % of organisms) above 1 mg/mL. This indicates that the samples did not cause significant mortality in the organisms tested during the 96 h period.

An LC50 above 1 mg/mL indicates that the concentration is not lethal to at least half of the exposed organisms. This is a positive indication of safety regarding the acute effects of the samples on the organisms analyzed.

Studies evaluating the toxicity of food products in adult zebrafish have been conducted, demonstrating the products' safety for consumption (*49-51*).

*Influence of bacterial cellulose application in smoothies*

The inclusion of bacterial cellulose from kombucha powder directly influenced the vitamin C content of this product, especially with acerola bacterial cellulose powder (Table 3).

The pH values are a safety parameter for food products. No significant difference in pH was found among the smoothies. Therefore, for this parameter, the addition of acerola, passion fruit, or green tea bacterial cellulose powder did not influence this characteristic. Regarding acidity, the formulations containing passion fruit and acerola bacterial cellulose were more acidic, which can be attributed to the powders' inherent acidity (Table 3). The combination of fermentation, bacterial cellulose composition, time, and additional ingredients may contribute to the observed differences in

smoothie acidity.

Analyzing 36 samples of smoothies made with different fruit pulps, acidity values ranged from 0.494 to 1.60 g of lactic acid per 100 g of sample (*52*).

A difference was also observed in the soluble solids content of the beverage developed without bacterial cellulose powder, which was 26.7 (ºBrix). In contrast, for the formulations containing kombucha bacterial cellulose powder, they presented values of 16 to 18 (ºBrix), probably due to the composition of the bacterial cellulose itself, which may have influenced the arrangement of the soluble molecules present in it.

In the research carried out by Gallina *et al.* (*52*), the soluble solids values for acerola, passion fruit, strawberry, and mango smoothies ranged from 12.1 to 18.7 (ºBrix), values similar to those found in this research.

The $a_w$ values for all formulations were 0.98, not statistically different from each other. Vitamin C had the highest content in formulations F1 and F2, which differed significantly from the other formulations at the $p<0.05$ level. This difference between vitamin C levels includes ingredients with the highest vitamin C content, such as acerola kombucha bacterial cellulose powder (1998.04 mg/100 g) and passion fruit bacterial cellulose powder (163.29 mg/100 g). Therefore, the inclusion of these powders, compared with the control smoothie, increases the content of this vitamin.

Only the F0 formulation showed a higher humidity value (11.11 %) than the other formulations.

Regarding the protein content of the smoothies, a significant difference ($p<0.05$) was observed between the smoothie without added bacterial cellulose powder ((2.31±0.43) %) and the others. The smoothie added with acerola bacterial cellulose powder had a protein value ((4.90±0.35) %), standing out from the other formulations prepared, followed by the smoothie added with passion fruit bacterial cellulose powder and the smoothie added with green tea bacterial cellulose powder. The kombucha bacterial cellulose powder is mainly composed of a cellulose matrix produced by the bacteria and yeast present in it. This matrix contains proteins that contribute to increased levels in smoothie formulations. A maximum protein content of 2.76 % was found in smoothies made with pineapple, watermelon, and mango (*53*).

Regarding the ash content of the smoothie beverages, there was a significant difference ($p<0.05$) between the smoothie without added bacterial cellulose powder and the other smoothies with added bacterial cellulose powder. Acerola bacterial cellulose powder presented a higher amount of minerals and inorganic compounds compared to passion fruit kombucha and green tea bacterial cellulose powders. Thus, by adding acerola bacterial cellulose powder to the smoothie, it is possible to increase the ash content (an indicator of mineral content) in the final beverage.

The lipid content in the smoothie beverage formulations did not differ significantly ($p>0.05$),

indicating that the addition of bacterial cellulose powder did not affect this component. Although the majority of bacterial cellulose is composed of cellulose and other carbohydrates, it is also possible to find lipids in smaller quantities. Therefore, this characteristic observed in smoothies is relevant and can be used as a nutritional appeal, making them even more attractive to people interested in consuming healthier foods with low lipid and caloric content.

The processing of the smoothies followed good handling practices, with total and thermotolerant coliform counts <3 MPN/g for all formulations (data not shown). Regarding mesophilic counts, counts of $10^2$ CFU/g were obtained. Given that the pulps used were not pasteurized and had a microbial load, it is reasonable to assume this count comes from the raw materials used to prepare the food, which is not considered high.

Treatment effects on the rheological properties of foods must be known for better process control (*54*). Understanding flow behavior is necessary to determine food viscosity.

When developing smoothies, one parameter to analyze is the food viscosity. Some authors include vegetables in their formulations that positively influence this parameter, such as pumpkin and carrots in a banana smoothie (*55*), or use a fruit mixture such as banana and melon smoothie (*56*).

In the formulations developed in this research, a mixture of fruits already established in gastronomy (strawberry and banana) was used, and the investigation focused on whether bacterial cellulose powders could also influence the final viscosity of the product.

During rheological analysis, the rheometer applies a known shear force to the smoothie and measures the fluid's response, including the shear rate and the resulting shear stress (Fig. 4).

Rheological analysis of smoothies provides insights into the flow behavior of this product. Rheological analysis is used to adjust the smoothie texture and consistency, optimize processing parameters, develop new products, or ensure the quality and consistency of the final product. Additionally, rheological analysis can help understand how ingredients, such as bacterial cellulose powder, affect smoothie properties and how they interact with other components.

The smoothie with acerola bacterial cellulose powder (Fig. 4) showed the most similar rheological behavior to the control (F0), followed by the smoothie with green tea bacterial cellulose. The smoothie containing passion fruit bacterial cellulose (F2) exhibited a very different rheological behavior from the other formulations, suggesting that this sample has higher viscosity than the others. It is noteworthy that the acerola residue may contain substances, such as pectin, which may have influenced the result obtained.

There are different types of non-Newtonian behavior, such as pseudoplastic, dilatant, and thixotropic, among others. The flow curve for a non-Newtonian fluid may have a descending linear shape (pseudoplastic), an ascending linear shape (dilating), or a curve with a conical shape, for

example. The smoothies developed in this research showed pseudoplastic behavior.

Regarding the toxicity analysis of the smoothies (Fig. 5), there was no toxic effect in response to sample administration, a result similar to that obtained for the isolated analysis of the powders. This suggests that the formulations did not cause immediate or obvious adverse effects in fish during the open-field test.

In all samples, no mortality was recorded after 96 h, with LC50 values >0.25 mg/mL. Therefore, the formulations appear to be safe for human consumption, with no toxicity that could compromise human health. Although several studies have evaluated the possible sedative effects of different foods on zebrafish, there is a need for more studies investigating the sedative effects and the mechanisms of action of smoothies in zebrafish, and this study is one of the pioneers in the field.

No study in the literature used this methodology to evaluate the toxicity of smoothies containing bacterial cellulose powder. Bacterial cellulose powders produced with acerola, passion fruit, and green tea byproducts have adequate physicochemical characteristics, significant levels of vitamin C, relevant proximate composition, and are microbiologically safe, with acerola bacterial cellulose powder standing out. These results offer significant opportunities for the food industry, providing consumers with nutritional and sustainable options, as well as for their application in the development of smoothies. It is worth noting that the product developed is relevant to an increasingly health-conscious consumer audience. Mainly used for health-related issues, older adults, or individuals with metabolic problems.

## CONCLUSIONS

The bacterial cellulose powders from acerola, passion fruit, and green tea showed promise, with suitable physicochemical characteristics, significant vitamin C contents, and microbiological safety. When added to smoothies, these powders significantly enriched the nutritional value of the products. Furthermore, FTIR and TG analyses provided valuable insights into the chemical changes in the smoothies resulting from the addition of bacterial cellulose, thereby improving our understanding of their composition and quality. Tests on zebrafish showed no adverse effects from the formulations, while toxicity tests confirmed the safety of the smoothies for human consumption, both pure and diluted. These results open exciting opportunities in the food industry, offering consumers nutritional and sustainable options.

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## ETHICS APPROVAL

The experimental procedures were approved by the Animal Use Ethics Committee of the Federal University of Ceará (CEUA-UFC), nº 1806202101.

## CONFLICT OF INTEREST

There is no conflict of interest.

## SUPPLEMENTARY MATERIAL

Supplementary material is available at www.ftb.com.hr.

## AUTHORS' CONTRIBUTION

R.S. Cruz carried out the kombucha and bacterial cellulose formulations and wrote the original draft of the manuscript. G.M. do Prado corrected and critically reviewed the manuscript. P.H.M. de Sousa participated in the interpretation of data and a critical review. N.M.P.S. Ricardo and D.H.A. Brito participated in the FTIR and TGA analyses. F.E.A. Magalhães and J.A. Furtado carried out zebrafish analysis. L.M.R. da Silva not only designed experiments but also revised and edited the manuscript. All authors approved the final version of the manuscript.

## ORCID ID

R.S. Cruz https://orcid.org/0000-0002-0480-3367
G. M. do Prado https://orcid.org/0000-0002-3008-7632
P.H.M. de Sousa https://orcid.org/0000-0001-7005-6227
N.M.P.S. Ricardo https://orcid.org/0000-0003-1849-5403
D.H.A. Brito https://orcid.org/0000-0003-2756-1820
F.E.A. Magalhães https://orcid.org/0000-0002-4924-2882
J.A. Furtado https://orcid.org/0000-0003-0146-0781

L.M.R. da Silva https://orcid.org/0000-0001-7302-401X

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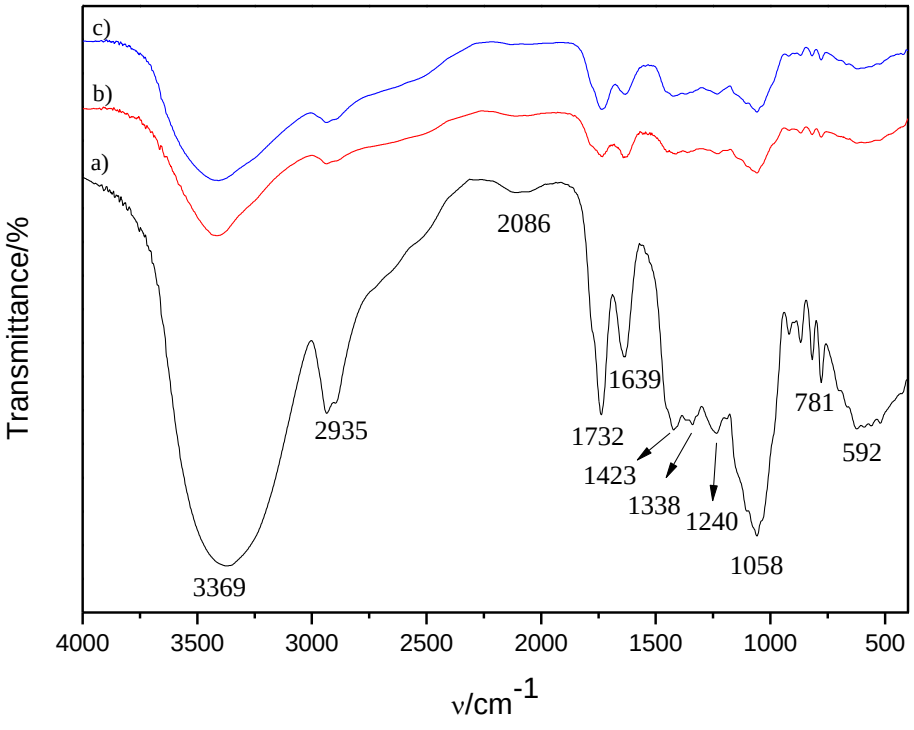


Fig. 1. FTIR spectra of acerola (a), passion fruit (b), and green tea (c) bacterial cellulose powder

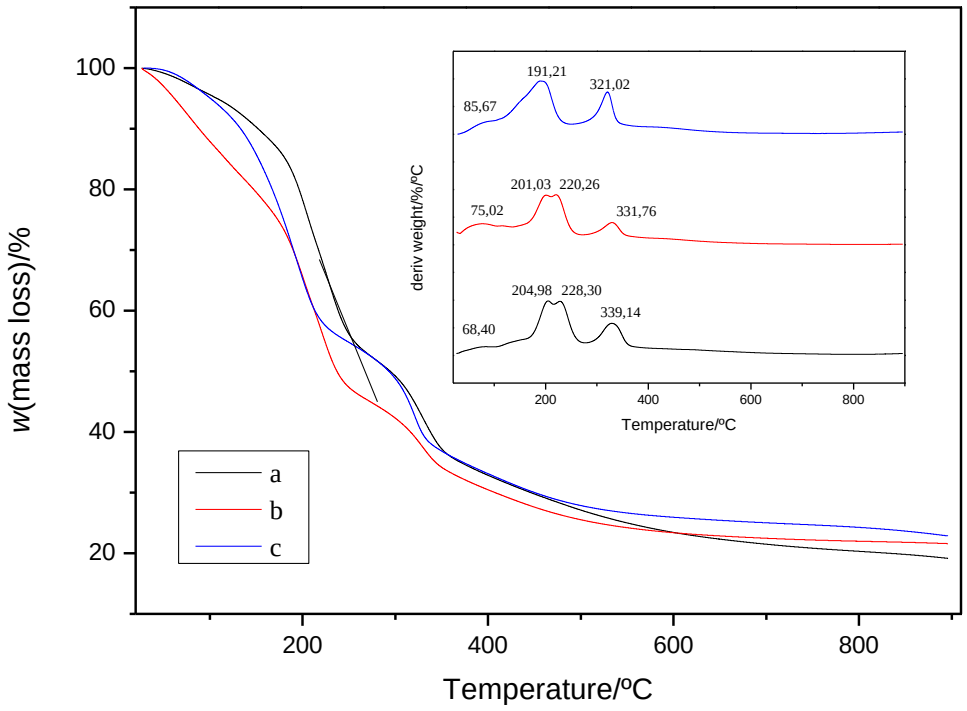


Fig. 2. Thermogravimetric analysis of acerola (a), passion fruit (b), and green tea (c) bacterial cellulose powders

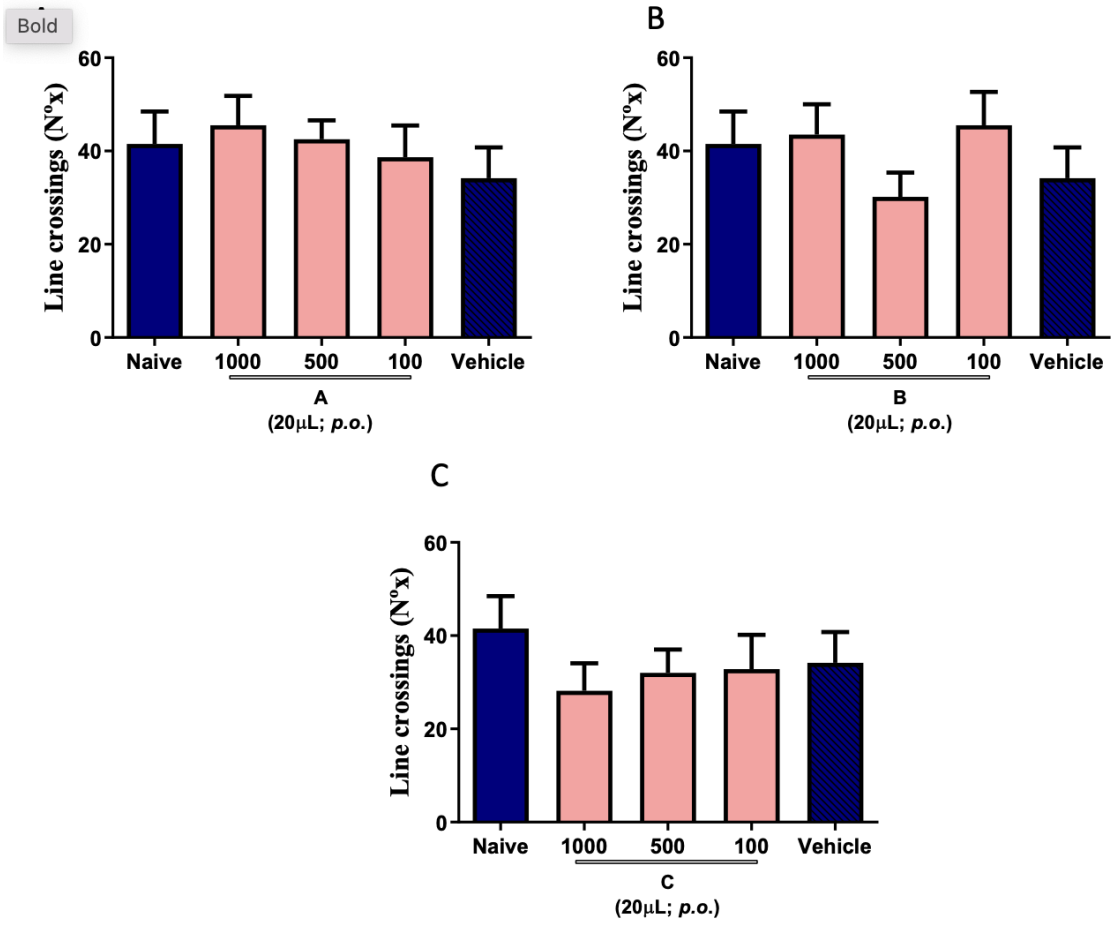


Fig. 3. Effect of: a) acerola bacterial cellulose powder, b) passion fruit bacterial cellulose powder, and c) green tea bacterial cellulose powder on the locomotor activity of adult zebrafish (*Danio rerio*) in the open field test. Crossing of lines referring to samples A, B, and C at concentrations 1000, 500, and 100 ppm. Naïve=untreated animals, p.o.=oral administration, vehicle=sterile distilled water (20 μL; p.o.). Values represent the mean±standard deviation of the mean for 6 animals/group (ANOVA followed by Tukey's test)

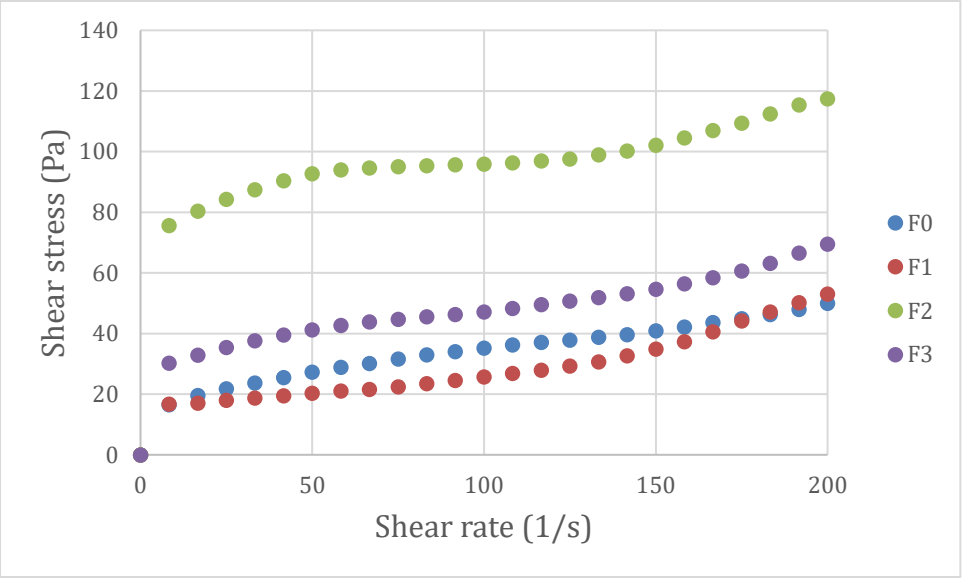


Fig. 4. Smoothie rheograms of formulations F0 (control), F1 (acerola bacterial cellulose), F2 (passion fruit bacterial cellulose), and F3 (green tea bacterial cellulose). Viscosity as a function of shear rate

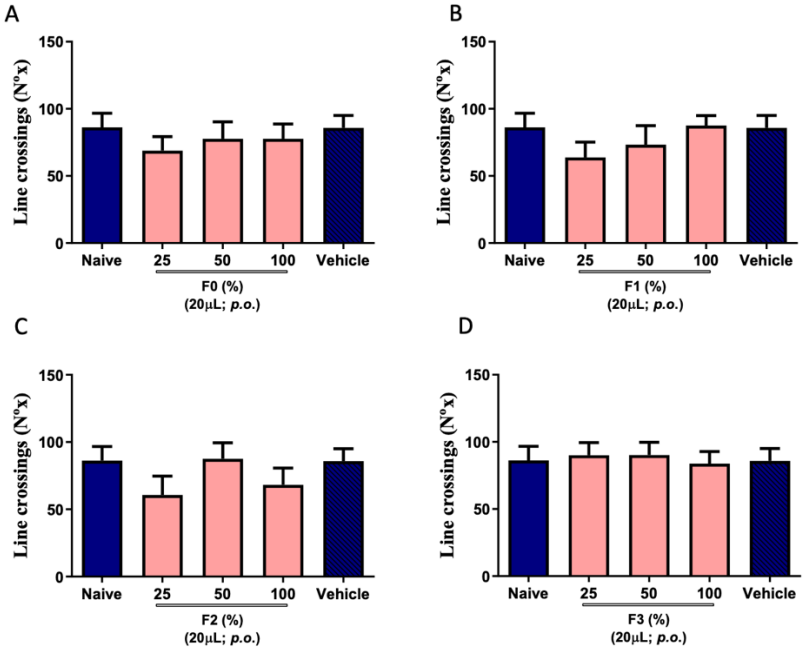


Fig. 5. Effect of formulations (F0, F1, F2 and F3) of smoothies on the locomotor activity of adult zebrafish (*Danio rerio*) in the open field test. Crossing of lines referring to the smoothie sample without the addition of bacterial cellulose powder (A); smoothie sample with the addition of acerola bacterial cellulose powder (B); smoothie sample with added passion fruit bacterial cellulose powder (C); smoothie sample with addition of green tea bacterial cellulose powder (D) at concentrations of 100, 50, and 25 %. Naïve=untreated animals, grandmother=oral administration, p.o.=oral administration,

vehicle=sterile distilled water (20 µL; p.o.). Values represent the mean±standard deviation of the mean for 6 animals/group (ANOVA followed by Tukey's test)

Table 1. Control parameters during the evaluated fermentation period

| *t*/day | Acerola | | Passion fruit | | Green tea | |
|---|---|---|---|---|---|---|
| | TSS/ºBrix | pH | TSS/ºBrix | pH | TSS/ºBrix | pH |
| 0 | (12.5±0.11) | (3.50±0.16) | (13.1±0.25) | (3.80±0.11) | (11.3±0.31) | (4.50±0.07) |
| 1 | (12.5±0.31) | (3.50±0.13) | (12.0±0.15) | (3.80±0.23) | (30.0±0.14) | (4.50±0.13) |
| 2 | (11.0±0.05) | (3.00±0.21) | (10.9±0.19) | (3.20±0.41) | (27.5±0.05) | (4.20±0.09) |
| 3 | (11.0±0.16) | (2.82±0.09) | (10.1±0.30) | (2.80±0.16) | (25.0±0.31) | (3.80±0.12) |
| 4 | (10.4±0.21) | (2.67±0.31) | (9.7±0.14) | (2.65±0.11) | (19.8±0.35) | (3.50±0.17) |
| 5 | (10.1±0.35) | (2.50±0.35) | (9.5±0.21) | (2.60±0.09) | (19.3±0.28) | (3.20±0.10) |
| 6 | (9.5±0.24) | (2.49±0.15) | (9.5±0.09) | (2.50±0.10) | (18.0±0.25) | (2.90±0.08) |
| 7 | (9.5±0.11) | (2.49±0.14) | (8.8±0.13) | (2.50±0.13) | (17.2±0.36) | (3.00±0.16) |

TSS=total soluble solids

Table 2. Physicochemical characterization of kombucha bacterial cellulose powders

| Parameter | Acerola bacterial cellulose | Passion fruit bacterial cellulose | Green tea bacterial cellulose |
|---|---|---|---|
| TTA/(mmol/L) | (3.19±0.30) $^{a}$ | (3.77±0.42)$^{a}$ | (4.15±0.22)$^{a}$ |
| pH | (2.77±0.04)$^{a}$ | (2.86±0.10)$^{a}$ | (2.90±0.00)$^{a}$ |
| *w*(vitamin C)/(mg/100 g) | (1998.04±50.75)$^{a}$ | (163.29±3.09)$^{c}$ | (393.46±4.17)$^{b}$ |
| TSS/ºBrix | (6.67±0.32)$^{a}$ | (6.13±0.06)$^{ab}$ | (8.13±0.06)$^{c}$ |
| *w*(protein)/% | (1.37±0.20)$^{a}$ | (1.39±0.19)$^{a}$ | (0.63±0.26)$^{a}$ |
| *w*(ash)/% | (2.58±3.31)$^{a}$ | (1.08±0.47)$^{a}$ | (2.19±0.20)$^{a}$ |
| *w*(lipid)/% | (0.56±0.00)$^{a}$ | (0.23±0.00)$^{a}$ | (0.6±0.00)$^{a}$ |
| $a_w$ | (0.22±0.00)$^{a}$ | (0.28±0.01)$^{a}$ | (0.24±0.01)$^{a}$ |

TTA=total titratable acidity, TSS=total soluble solids

Table 3. Physicochemical, proximate, and vitamin C analyses of smoothie beverages with and without the addition of bacterial cellulose powder

| Parameter | Smoothie | | | |
|---|---|---|---|---|
| | F0 | F1 | F2 | F3 |
| pH | (4.5±0.60)$^{a}$ | (4.2±0.12)$^{a}$ | (4.2±0.25)$^{a}$ | (4.3±0.27)$^{a}$ |
| TTA/(mmol/L) | (2.98±0.02)$^{a}$ | (3.9±0.10)$^{a}$ | (4.5±0.04)$^{ab}$ | (5.10±1.01)$^{bc}$ |
| TSS/°Brix | (26.7±0.12)$^{a}$ | (17.00±0.23$^{b}$ | (18.00±0.03)$^{b}$ | (16.00±0.19)$^{b}$ |
| $a_w$ | (0.98±0.001)$^{a}$ | (0.98±0.00)$^{a}$ | (0.98±0.001)$^{a}$ | (0.98±0.002)$^{a}$ |
| *w*(vitamin C)/(mg/100 g) | (51.16±0.23)$^{a}$ | (275.07±0.11)$^{b}$ | (145.63±0.04)$^{c}$ | (74.00±0.01)$^{ad}$ |
| *w*(humidity)/% | (11.11±0.24)$^{a}$ | (6.41±0.16)$^{b}$ | (7.35±0.02)$^{bc}$ | (7.89±0.32)$^{bc}$ |
| *w*(protein)/% | (2.31±0.43)$^{a}$ | (4.90±0.35)$^{b}$ | (3.57±0.13)$^{c}$ | (2.64±0.33)$^{cd}$ |
| *w*(ash)/% | (0.72±0.55)$^{a}$ | (3.01±0.23)$^{b}$ | (1.78±0.02)$^{c}$ | (2.61±0.65)$^{cd}$ |
| *w*(lipid)/% | (0.25±0.28)$^{a}$ | (0.58±0.06)$^{a}$ | (0.44±0.22)$^{a}$ | (0.35±0.14)$^{a}$ |

TTA=total titratable acidity, TSS=total soluble solids, F0=control, without added bacterial cellulose powder, F1=smoothie with acerola bacterial cellulose powder, F2=smoothie with passion fruit bacterial cellulose powder, F3=smoothie with green tea bacterial cellulose powder. Mean values followed by a capital letter in the column and a lowercase letter in the row do not differ from each other ($p>0.05$), determined using Tukey's test

## Supplementary material

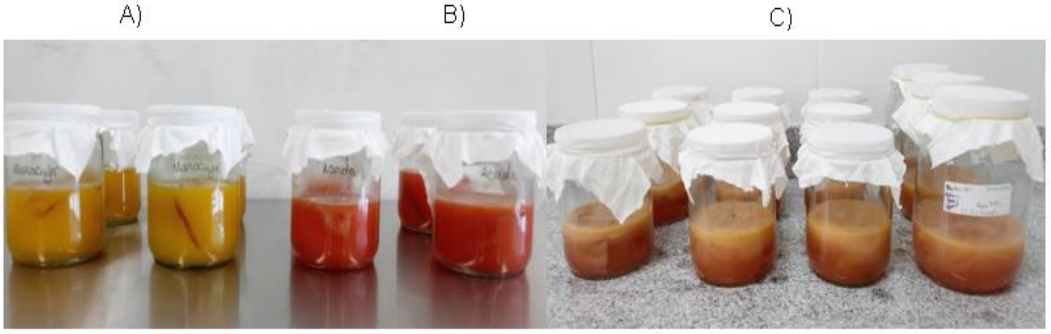


Fig. S1. Initial fermentation process - containers with: a) passion fruit, b) acerola, and c) green tea byproducts at rest

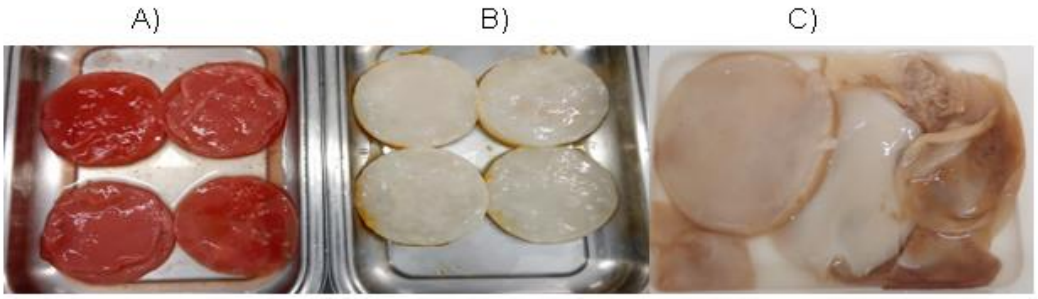


Fig. S2. Obtaining bacterial cellulose from: a) acerola, b) passion fruit, and c) green tea substrate

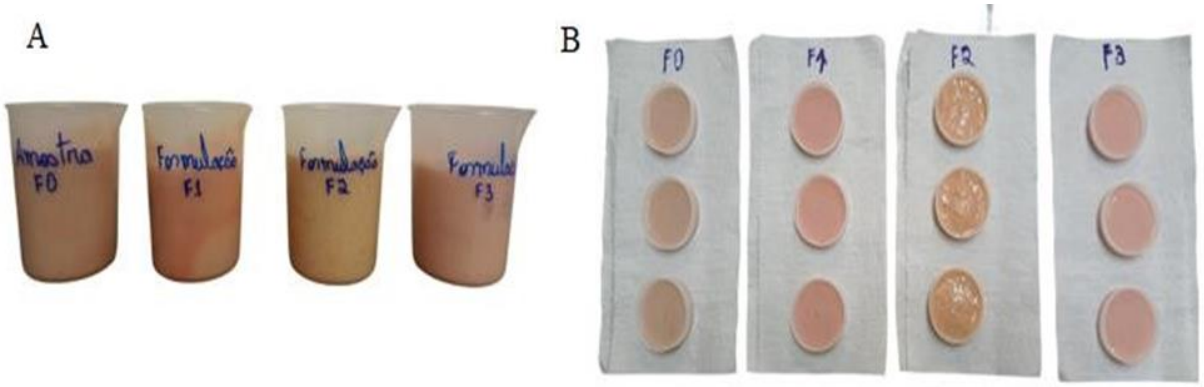


Fig. S3. Processing of beverages with the addition of the developed bacterial cellulose powders: a) smoothie without the addition of bacterial cellulose powder (F0), and with the addition of bacterial cellulose powder from acerola (F1), passion fruit (F2), and green tea (F3) kombucha, and b) homogenized smoothie formulations