



On the microstructural anisotropy and mechanical properties of bacterial nanocellulose obtained by static culture

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ARTICLE INFO

Keywords:

Bacterial NanoCellulose (BNC)
Static culture
Microstructure
3D anisotropy
Scanning electron microscopy (SEM)
Stimulated emission depletion microscopy (STED)
Mechanical testing
Evolutionary algorithms (EA)
Finite element (FE) analysis

ABSTRACT

The microstructural organization of bacterial nanocellulose is still not completely understood. Although its morphological and mechanical anisotropy is commonly recognized, it remains unclear whether the forces stabilizing the cellulose fiber network are comparable in different planes. In this study, we characterized the microstructure of statically produced BNC according to its horizontal and vertical planes. The tensile mechanical response in both planes was also investigated and complemented by finite element modeling.

The intrinsic 3D anisotropy of BNC microstructure, obtained by static culture, was demonstrated by scanning electron microscopy, even without a stratified layered microstructure. A mechanism based on swelling-induced stretching of BNC in the vertical direction is suggested to explain the observed fiber reorganization during membrane growth: high morphological anisotropy of the fibers in the upper surface partially dissipates towards depth. Moreover, the distinct tensile mechanical response observed for both planes (Young's modulus: 1.9 MPa horizontal and 0.9 MPa vertical) supports the 3D anisotropy of static cultured BNC. However, the similar maximum tensile strength (0.39–0.41 MPa) suggests that fiber networking and cohesion are similar in orthogonal orientations. The similar cross-linking density in both planes ($5.8\text{--}6 \times 10^8$ counts/mm³) determined by stimulated emission depletion microscopy suggests the same conclusion.

1. Introduction

Synthesized in its pure form by a variety of microorganisms from different genera, *Gluconacetobacter*, *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Rhizobium*, etc., bacterial nanocellulose (BNC) consists of a unique 3D network of nanofibers assembled by a supramolecular mechanism [1–3]. In a static regime, BNC production occurs at the air-liquid interface, a pellicle thickening downwards into the culture medium; the bacteria trapped in the sinking BNC matrix become inactive or die due to the depletion of oxygen and nutrients [4–6]. The secreted linear stacked β -1,4-glucan chains self-assemble through intra- and intermolecular hydrogen bonding into uniaxial parallel-arranged fibers, forming hierarchically ordered structures from protofibrils, consisting of

16 glucan chains (2–3 nm width) to bundles, sheets and ribbon-like structures with varying widths (20–120 nm) [7]. On a wider scale, these fibrous structures are interconnected through crosslinks, either permanent (fiber ramifications) or non-permanent (van der Waals forces, including hydrogen bonds), tightly holding the BNC membrane together. This hierarchical supramolecular assembly mechanism results in a highly porous macroscopic 3D network of nanofibers exhibiting a multiscale pore size ranging from the nanometer scale to up to tens of microns [8].

Recent reports have uncovered and reviewed the BNC structure-property relationships [7,9,10]. Despite the identical chemical structure compared to its plant-derived counterpart, BNC exhibits outstanding mechanical properties [11]. The high mechanical strength

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<https://doi.org/10.1016/j.ijbiomac.2025.148114>

Received 15 April 2025; Received in revised form 16 September 2025; Accepted 3 October 2025

Available online 4 October 2025

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of BNC has been attributed to a combination of several interconnected factors. The experimentally demonstrated or modeled axial Young's modulus (E) of Cellulose type I crystal structure is within 110–220 GPa and tensile strength (σ^u) of 7.5–7.7 GPa [12]. The mechanical performance of a single nanofiber is characterized by a high Young's modulus within 53–114 GPa [13–16]. The high aspect ratio of the nanofibers results in a high degree of polymerization (DP) (4000–10,000) [11,17,18]. Moreover, the proposed high horizontal orientation of the static cultured BNC and the three-way branching points are all associated with the superior mechanical performance of BNC [6,8,19].

Three-way branching points have been described as strong and permanent physical crosslinks at crystalline junction zones, resulting from cell division and movement during cellulose secretion [6,10,19]. These branching points, unifying BNC as a one-piece material, have been pointed out as the main contributors to BNC elasticity. In contrast, transient contact points, described as transient bonds based on fiber-fiber weak interactions (hydrogen bonding or Van der Waals forces), are thought to contribute to the viscoelastic behavior of the BNC [10]. Mechanical interlocking due to the entanglement of the randomly generated fibers certainly also plays a relevant contribution to the viscoelastic behavior of BNC.

Also, the microstructural organization of BNC, influenced by the bacterial strain, production regime (static or agitated conditions) and production rate, has a profound impact on its mechanical performance [9]. A great variety of mechanical properties of native (hydrated) BNC can be found in the literature, with apparent Young's modulus varying within 0.34–14.2 MPa and breaking strengths oscillating from 0.15 to 2.2 MPa [20–22]. Recently, Takayama and Kondo [10] demonstrated a strong positive linear relation between cross-linking density, the apparent elastic modulus and breaking strength of BNC, rather than the cellulose content [10,23]. Besides cross-linking density and cellulose content, the microstructural organization of BNC networks can impart different mechanical properties [9]. The existence of anisotropically layered structures on static cultured BNC has been reported by several authors, presenting distinct interlayer distances [19,24–28].

In a recent study, Gromovych et al. [9] attempted to unravel the origin of anisotropy and of the layered structure of BNC produced by *G. hansenii*, in particular, in planar and spherical configurations. The authors suggested that the development of layered sheets on BNC is related to the formation of fibrous density gradients when the synthesis rate exceeds a certain threshold. The formation of fibrous gradients leads to a restriction of bacterial cell movement and entrapment in the fibrous mesh, resulting in the production of dense sheets and separation into layers. Conversely, the authors argued that when BNC production is slow, the density gradients can be dissipated and the microstructural organization tends to be isotropic [9]. The authors also demonstrated that freeze-dried BNC exhibiting anisotropic layered microstructure possesses a higher Young's modulus and lower elongation (Young's modulus of 6.5 ± 1.5 GPa and 1.5 % elongation at break) when compared to freeze-dried isotropic BNC, being softer and more deformable (Young's modulus of 0.06 ± 0.02 GPa and 6 % elongation at break) [9]. In addition to the valuable insights reported on the structure–property relationships of BNC, which will certainly contribute to its optimized valorization, Murugarren et al. [29] have demonstrated the ability to control fiber directionality during the cultivation process through static cultivation and spatial confinement of bacteria, thereby opening new opportunities for the production of BNC membranes with tailored properties.

All the gathered knowledge in recent years on BNC has provided important insights into the fundamental understanding of its microstructural organization and structure–property relationships. However, a critical examination of the recently published literature reveals unresolved questions regarding the influence of static culturing on the microstructural organization of BNC and its implications for anisotropic mechanical behavior, which are particularly relevant when biomedical applications are envisioned. Specifically, it remains unclear how the

microstructural organization, particularly the fiber orientation and cross-linking density, influences the cohesive forces and mechanical properties across different planes of static-cultured BNC membranes. This study hypothesizes that the anisotropic layered structure of BNC results in distinct cohesive forces and mechanical properties in orthogonal planes, which can be tailored to mimic the three-dimensional anisotropy of biological tissues for tissue engineering and regenerative medicine. To test this hypothesis, we conducted a comprehensive multiscale characterization of the orthogonal planes of *Komagataeibacter xylinus* static-cultured BNC by (1) comparing fiber orientation and cross-linking density across orthogonal planes, using advanced microscopy techniques, such as scanning electron microscopy (SEM) and stimulated emission depletion (STED) microscopy; (2) evaluating the cohesive forces and stiffening behavior through tensile mechanical testing in orthogonal planes; and (3) correlating these properties with numerical simulation modeling to elucidate structure–property relationships. These specific comparisons would provide insights into whether cohesive forces and mechanical properties, such as stiffness and ultimate tensile strength, vary significantly across different orientations of the BNC membranes, paving the way for optimized static culturing and application-oriented post-processing for biomedical applications.

2. Materials and methods

2.1. Materials and reagents

The bacterial strain *Komagataeibacter xylinus* ATC 700178 was purchased from the American Type Culture Collection. Agar was acquired from Acros Organics. Molasse and Corn Steep Liquor (CSL) were gently supplied by RAR (Refinarias de Açúcar Reunidas, S.A.; Portugal) and COPAM (Companhia Portuguesa de Amidos, S.A.; Portugal), respectively. Ammonium sulfate was acquired from Panreac and ethanol from Fisher Chemical. Disodium phosphate di-hydrated ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) was purchased from Labkem and citric acid from Panreac. Cellulase (Celluclast® 1.5 L) was acquired from Novozymes. Carbotrace 680 was acquired from Ebba Biotech.

2.2. Bacterial nanocellulose production

2.2.1. Bacterial strain and inoculum preparation

BNC production was carried out with *Komagataeibacter xylinus* ATCC 700178 from the American Type Culture Collection, under static culture fermentation. The bacterial stock was maintained cryopreserved at -80°C in liquid Hestrin-Schramm culture medium (HS) with 20 % (w/v) of pure glycerol until use. The cryopreserved cells were thawed at room temperature and revived by transferring them into fresh Hestrin-Schramm (HS) culture medium [30] and incubated at 30°C for 24–48 h under static conditions (XU980, C3000, France Etuves) to promote recovery and initial growth. About 200–300 μL of the revived culture was withdrawn and streaked onto HS-agar (20 g/L) plates at 30°C for 3 to 4 days under humid conditions until colonies were well established and ready for use. Inoculum preparation and static fermentation were both performed in an optimized medium [31] containing molasses (MOL) and Corn Steep Liquor (CSL) as carbon and nitrogen sources, respectively, with some modifications. All the mediums used for pre-inoculum preparation and fermentation culture were autoclaved at 121°C and 1 bar for 20 min before use.

ATCC 700178 cells were grown in 1L conical flasks containing 100mL of a MOL-CSL-based medium, comprising the following components (in g/L): molasses (20.0 of total sugars), CSL (7.0 of total protein content), citric acid (1.26), and disodium phosphate (3.39). The pH was adjusted to 5.5. The culture was incubated for 2 days at 30°C under static conditions. Afterward, the culture was vigorously shaken to remove active cells entrapped within the cellulose pellicle, and the inoculum (10 % v/v) was transferred to 500mL conical flasks containing 112.5 mL of MOL-CSL medium (composition described above)

supplemented with 0.025 % (v/v) of cellulase (pH set to 5.5). The inoculum was maintained in a rotary shaker (MaxQ 481R HP, Thermo Scientific) at 30 °C under orbital agitation (160 rpm) for 20 h.

2.2.2. Static culture fermentation and BNC purification

BNC static culture fermentation was performed in plastic containers (24 cm length x 14 cm width x 9.5 cm height; 336 cm²) containing 850 mL of MOL-CSL based fermentation medium with the following composition (in g/L): molasses 40.0 of total sugars, CSL 7.0 of protein content, ammonium sulfate 0.5 % (w/v), ethanol 1.5 % (v/v), disodium phosphate dihydrate (3.39) and citric acid (1.26). Inoculum 0.5 % (v/v) was transferred to a fresh MOL-CSL-based fermentation medium and incubated for 24 days at 30 °C under static conditions. Three independent cultures were performed to obtain membranes for subsequent characterization analysis. After cultivation, BNC membranes were collected and washed with distilled water at room temperature to remove culture medium residues, followed by exhaustive washing in 0.1 M NaOH solution (changed daily) until bleached membranes were obtained. The purified BNC membranes (featuring approximately 35–38 mm thickness) were sterilized by autoclaving for 20 min at 120 °C and 1 bar and stored at 4 °C until characterization. Given the high thickness of the obtained membrane, bacteria were not fully removed through this methodology. Although the presence of bacteria may, to some extent, influence the mechanical characterization results, a more thorough washing to remove bacteria could alter 3D microstructural features. Therefore, we intentionally avoided exhaustive washing to preserve the native microstructure and its influence on the mechanical response, which was the primary focus of this work. To avoid the effect of compression during membrane manipulation, all test specimens were obtained through laser cutting and were left in water until testing.

2.3. Morphology characterization

2.3.1. Scanning electron microscopy (SEM)

To assess the microstructure organization of the BNC network along the BNC membrane horizontal surface (XY plane) and a vertical plane (X,Y-Z) (as described in the results section, the distribution of fibers on the horizontal XY plane is isotropic; therefore, the morphology of vertical planes – e.g. XZ, YZ or any other vertical plane – is similar). For practical reasons, in some experiments, it is hard to state unequivocally whether a XZ or YZ plane is being observed. Given that morphologically (or mechanically) those planes are equivalent, they are identified, for simplicity, as X,Y-Z planes throughout this work. The membranes were first sliced in the XY plane into three visually distinct zones: a 5 ± 1 mm thick surface layer, slightly translucent air-liquid interface; a 20 ± 1 mm thick, homogeneous middle region; and a 10 ± 1 mm thick bottom layer, more translucent region (Fig. 1a). Then, from each of the three membranes, 10 mm prism-shaped samples were obtained from the three

distinct regions within each BNC membrane using CO₂ laser cutting (Fig. 1a). Afterward, BNC samples were quickly frozen in liquid nitrogen, and cross-sectional XY and X,Y-Z faces were exposed by fracturing BNC before lyophilization (Fig. 1b).

In addition, to assess the stress-transfer mechanisms of the BNC network along different BNC growth planes (XY and X,Y-Z planes), specimens subjected to uniaxial tensile mechanical tests obtained immediately after rupture were quickly frozen in liquid nitrogen and lyophilized. Before SEM imaging, all the samples were mounted on aluminum pin stubs and sputter-coated with a thin layer of gold (50 Å). To assess microstructural variations, BNC specimens from distinct membrane regions were imaged in orthogonal planes at multiple spatially interspaced locations across the full thickness and surface area. Top-view images of tensile-deformed BNC specimens from horizontal (XY) and vertical (X,Y-Z) planes were acquired near the rupture site to assess fiber reorganization in response to mechanical loading. Imaging was performed using a Vega 3 SBH system (TESCAN) at 15 kV accelerating voltage and 4 mm working distance, operating under high vacuum conditions.

2.3.2. Stimulated emission depletion microscopy (STED)

The 3D data for quantitative analysis of BNC morphological organization, namely cross-linking density across horizontal (XY) and vertical (X,Y-Z) BNC planes, were obtained with STED. First, 10 mm prism samples were obtained from the middle homogeneous region (corresponding to approximately 57.1 % of the entire membrane thickness) (Fig. 1a) of three independently cultured BNC membranes using CO₂ laser cutting. Then, thin BNC pellicles (1 mm thick) were cut from orthogonal planes of these 10 mm prism samples using the same laser-cutting method (Fig. 1b).

The 1 mm-thick BNC pellicles were then stained with Carbotrace 680 (Ebba Biotech, Sweden), a red optotracer reliably used for labeling cellulose. Briefly, the BNC pellicles were incubated with Carbotrace 680 (dilution of 1:100) for 24 h at 4 °C to allow a bulk and homogeneous staining of the fibers. The stained samples were then rinsed with ultrapure water to remove the non-bound tracer and observed with a Leica STELLARIS 8 STED FALCON system using an HC PL APO 86×/1.20 W motCORR STED white objective (Leica Microsystems, Germany). The measurements were done with excitation by a pulsed white light laser (set at 85 % of full power) turned at 633 nm (3 %) and depletion with a 775 nm laser at 77 % of full power; the emission detection was done with a HyD X in counting mode in the range of 645–720 nm. The STED system was automatically aligned and calibrated with internal routines of the system. Tau STED filter was applied with the following parameters: tauBackground on, tauStrength 100, and Denoise 50. Image acquisition was performed at 10–15 µm depth from the top surface of the pellicle to avoid heat-affected zones resulting from CO₂ laser cut. 3D imaging was performed in 20.8 µm × 20.8 µm × 5.11 µm regions by collecting series

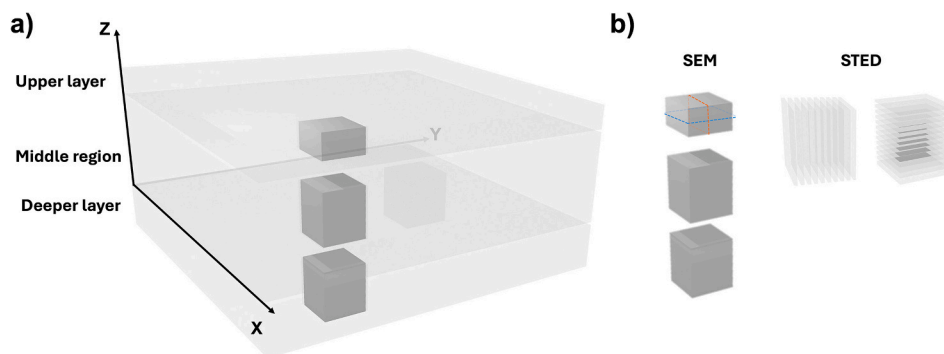


Fig. 1. Schematic representation of static BNC characterization procedure using SEM and STED microscopy. BNC sectioning in the XY plane to isolate distinct regions (a), followed by prism sample extraction using CO₂ laser cutting for subsequent SEM and STED analysis; sample preparation for SEM and STED with the analyzed planes is highlighted (b).

of 2D images acquired in 1024×1024 -pixel format $\times$ 31 frames (20.3 nm $\times$ 20.3 nm $\times$ 170 nm voxel size). For each BNC plane (XY and X,Y-Z), three biological replicates from independently cultured membranes were prepared and for each replicate, data were collected from five spatially interspaced, randomly selected points across the entire sample, totaling 15 multiple sections for each plane.

2.4. Image processing and analysis

Following analysis of the multiple acquired images for assessing microstructural variations, a representative SEM image was selected from each orthogonal plane and a distinct membrane region for detailed analysis using the *Orientation J* plugin on Fiji/ImageJ software (version 1.54f). Fiber orientation color map, orientation distribution ($^{\circ}$), the dominant direction ($^{\circ}$) and associated coherency (an index between 0 and 1, from random orientation-isotropy to perfect alignment-anisotropy) were determined for each orthogonal plane and distinct BNC membrane region to assess microstructural organization of the BNC network across different planes [32,33].

The acquired STED images were deconvolved in Huygens Professional software (Scientific Volume Imaging, Hilversum, Netherlands, Windows SO). First, misalignment of the sections due to thermal fluctuations was automatically corrected using the Object Stabiliser module of Huygens Pro and then the corrected Z-stacks were restored by a classical Maximum Likelihood Estimation deconvolution algorithm adapted for STED images applying express mode using the standard deconvolution parameters and a theoretical point spread function calculated based on the microscopic parameters. To reduce the file size for the image analysis, the images were then exported to TIFF format and, using Fiji/ImageJ software (version 1.54f, Windows OS), they were downsampled $4\times$ to a 256-pixel format (XY) while retaining the depth of 31 stacks (frames). In Fiji/ImageJ, an automatic brightness/contrast was applied to better visualize the 3D fiber network before analyzing it using SOAX software (as described ahead). The images were also saved as .tiff extensions. No explicit binarization or additional global noise removal filters were applied to the images before loading into SOAX for fiber tracing, as the software's internal parameters were subsequently optimized for segmentation and fiber tracing from the grayscale isotropic images, as described ahead.

The extraction of the fiber network structure and quantification of the network junctions were performed using SOAX (version 3.7.0, Windows OS), an open-source software used to trace the centerlines of 3D biopolymer networks accurately [34]. Before analysis, given the differing voxel spacing (see previous section) in the Z-axis compared to the X and Y-axes, the rescaled images were opened in SOAX and a rescaling step was performed to produce isotropic images suitable for analysis. For this purpose, a calculated value of 8.3620 (from the voxel spacing values described in the previous section) was applied for the Z-axis spacing relative to the XY-axis before saving as isotropic images (also as TIFF extensions). These isotropic images were opened again in SOAX for analysis.

For segmentation, the default parameter settings in SOAX were used, except for the following: since the edited image was isotropic, the background Z/XY ratio was set to 1. Init z (initial Z-axis spacing relative to the XY-axis measurements) was either selected or deselected on a case-by-case basis to optimize the initial placement of centerlines. Also, when needed, the snake point spacing was increased from 1 to a value of up to 3 pixels (to control the resolution of the extracted centerline) and the change threshold (to help distinguish between the background and the structures of interest) from 0.1 to 0.3 pixels. Snake evolution was performed with 10,000 iterations, a value of 100 for the check period and the iterations per press; also, a minimum snake length threshold of 0.1 pixels was used to avoid tracing short, noise-induced segments.

The default conditions were used for considering a junction point: an overlap threshold of 1 pixel (defines the distance threshold at which snakes are considered overlapping), a grouping distance threshold of 4

pixels (specifies the maximum distance that two T-junctions formed after snake evolution can be clustered into one clustered junction for grouping) and a minimum angle for SOAX linking of 2.1 (the minimum angle between two snake branches in a clustered junction to be grouped).

Following the centerline's initialization and evolution, they were cut and grouped. The centerlines' information (3D coordinates and the junction points) was saved as a text file. Finally, the crosslink density (cross-linking points/junctions per volume) was estimated using a total of 15 datasets obtained from three biological replicates for each orthogonal BNC plane (XY and X,Y-Z).

2.5. Mechanical characterization: uniaxial tensile test

The mechanical properties of BNC were assessed through uniaxial tensile tests in orthogonal directions: the XY and X,Y-Z planes. For this effect, 3 mm-thick BNC pellicles were cut from the orthogonal planes of three biological membrane replicates using a CO₂ laser. From these, dogbone-shaped test specimens (ISO 37-4) were cut using a CO₂ laser and a computer-aided graphical design. The X,Y-Z dogbone specimens were cut across the full thickness of the BNC membranes, exposing the homogeneous middle region used for crosslinking quantification as the gauge section, where deformation occurs, thus enabling correlation between microstructural and mechanical properties. The thickness of each sample was measured using a digital caliper (OMV-25MX; Mitutoyo, Kanagawa, Japan; resolution: 0.001 mm). All the specimens were maintained in water until testing to prevent variations in moisture content. Both ends of the test specimens were protected with paper strips before fixation on tensile grips to prevent damage and sample slipping during experiments.

Uniaxial tensile tests were conducted in a texture analyzer, TA.TX Plus Texture Analyzer, equipped with a 5 N load cell and the Exponent - version 6.1.16 software (Stable Micro Systems, Surrey, UK). The cross-head speed was set to 10 mm/min. Fifteen measurements were performed for each BNC plane, comprising five specimens per biological replicate from three independent cultures. From the obtained stress-strain curves, the apparent Young's modulus (MPa), ultimate tensile stress (MPa), and elongation at break (%) were calculated. Young's modulus was calculated using the slope of the linear region of the stress-strain curve, according to several reports [21,22,35].

2.6. Identification of BNC constitutive law using finite element (FE) analysis and evolutionary algorithms (EA)

To identify the most suitable model to describe the tensile behavior of BNC, MCalibration 5.1.2 software (Veryst Engineering, Needham, Massachusetts, USA) was employed in this work. Experimental true stress-strain ($\sigma - \epsilon$) data obtained from uniaxial tensile tests in the XY and X,Y-Z directions served as input for the model calibration. The objective function for optimization was defined as the mean squared difference (MSD) between numerical and experimental data ($\sigma - \epsilon$). Parameter search encompassed an extensive optimization process, beginning with the generation of an initial population (binary notation) of potential solutions, randomly formed, followed by a refinement phase using EA provided by MCalibration. Before the optimization process, key evolutionary parameters, such as the population size n , the crossover rate S_c , and mutation rate S_m , were fine-tuned to ensure robust calibration (Table 1), using a stopping criterion based on a maximum

Table 1
Evolutionary parameters used in the optimization method.

Population size (n)	1000
Crossover rate (s_c)	0.95
Mutation rate (s_m)	0.05
Maximum number of generations (n_{lim})	100

number of generations n_{lim} . The process was then carried out using the genetic operators of selection, crossover, and mutation, following a methodology that enabled the identification of hyperelastic coefficients that best align the numerical results with the experimental true stress-strain curves. Among the tested models, the Holzapfel-Gasser-Ogden (HGO) model emerged as the most accurate one in replicating experimental mechanical behavior for both loading directions. The adoption of a hyperelastic model is adequate in these conditions since the material's viscoelastic mechanisms were not visible in the time span of the tensile test.

2.6.1. Holzapfel-Gasser-Ogden model

The Holzapfel-Gasser-Ogden (HGO) model is widely used to replicate the anisotropic behavior of hyperelastic materials, assuming near-incompressibility [36], common in soft biological tissues. Although BNC is known to exhibit a viscoelastic response, the experimental tests were conducted in a time span that hindered time-dependent phenomena, allowing the application of the HGO model. This hyperelastic formulation is accomplished through a strain-energy function ($\bar{W}$), often expressed in terms of the invariants of the two Cauchy-Green deformation tensors [36,37]. The HGO model combines isotropic and anisotropic components in the strain energy density function to account for the bulk behavior of the matrix material and the contributions of fiber orientation. For tissues with perfectly aligned fibers, the strain-energy function of the HGO model (in its analytical form) can be expressed according to Eq. 1 [37,38]:

$$\bar{W} = \frac{G}{2}(\bar{I}_1 - 3) + \frac{k_1}{2k_2} \left[\exp\left(\frac{k_2}{9}(\bar{I}_1 - 3)^2\right) - 1 \right] + \frac{K}{2} \left(\frac{(J^{\text{el}})^2}{2} - \ln J^{\text{el}} \right) \quad (1)$$

with G standing for the material shear modulus, $\bar{I}_1$ for the first invariant of the isochoric part of the left Cauchy-Green deformation tensor (e_{ij}), k_1 for a material property (units of stress) that relates to fiber stiffness, k_2 for a dimensionless material parameter that expresses the fiber nonlinear behavior, N for the number of fiber families, $\bar{I}_{4(i)}$ for the anisotropic (reduced) pseudo-invariant allowing to compute the material's overall stretch, K for the bulk modulus, and J^{el} for the elastic volume ratio. The initial stiffness of the isotropic matrix is directly related to materials with incompressible or nearly incompressible behavior ($C_{10} = \frac{G}{2}$). The material's compressibility (D) is inversely proportional to the bulk modulus (K). The anisotropic pseudo-invariant is computed in the form $\bar{I}_{4(i)} = \mathbf{a}_i \cdot (\mathbf{C} \mathbf{a}_i)$, where $\mathbf{a}_i$ is the fiber direction and $\mathbf{C}$ the right Cauchy-Green deformation tensor. The definition $\bar{I}_{4(i)}$ considers dispersion using the parameter κ , resulting in $\bar{I}_{4(i)} = \kappa I_1 + (1 - 3\kappa) \mathbf{a}_i \cdot (\mathbf{C} \mathbf{a}_i)$, where $I_1 = \text{tr}(\mathbf{C})$, and $1 - 3\kappa$ adjusts the weight of the perfect alignment contribution $\mathbf{a}_i$ versus the isotropic contribution. The dispersion parameter κ (within the interval $0 \leq \kappa \leq 1/3$) determines whether fibers are mostly aligned ($\kappa = 0$), randomly scattered ($\kappa > 0$), or entirely dispersed ($\kappa = 1/3$) [39].

When an anisotropic component accounts for multiple fiber families, the strain energy density function [36–38] can be written according to Eq. 2, with N representing the number of fiber families contributing to the anisotropy.

$$\bar{W} = \frac{G}{2}(\bar{I}_1 - 3) + \frac{k_1}{2k_2} \sum_{i=1}^N \left[\exp\left(k_2(\bar{I}_{4(i)} - 1)^2\right) - 1 \right] + \frac{K}{2} \left(\frac{(J^{\text{el}})^2}{2} - \ln J^{\text{el}} \right) \quad (2)$$

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.3.0 software. Results were expressed as the mean $\pm$ SD. Data normality and homogeneity of variances were tested using the Shapiro-Wilk and F

tests, respectively, with a significance level of $p < 0.05$. Based on these results, appropriate statistical tests for comparisons between two groups were selected as follows: data following a normal distribution with equal variances was compared by a two-tailed unpaired t -test; data following a normal distribution but with unequal variances was analyzed by a two-tailed unpaired t -test with Welch's correction; data not fitting a normal distribution was analyzed by the non-parametric two-tailed Mann-Whitney test. The confidence level was set at 95 % for all statistical analysis and exact p -values are reported in the Results and Discussion section.

3. Results and discussion

Considering the potential of BNC for the development of advanced functional materials for a wide range of applications [40–44], a fundamental understanding of its intrinsic microstructural organization and structure-property relationship is critical for targeted-oriented production and application. In this work, we focused on the characterization of the 3D microstructure of statically produced BNC membranes and how it translates into their mechanical properties. The BNC membranes studied in this work exhibited a water content (%) of 99.1 ± 0.02 , a crystallinity index (CrI%) of 88, a molecular weight of 392 ± 12 and a degree of polymerization (DP) of 2419 ± 74 , which were thoroughly characterized elsewhere [45].

A comprehensive analysis was conducted to evaluate morphological variations—such as fiber arrangement and cross-linking density—alongside the mechanical response and microstructural reorganization under uniaxial tensile loading. These assessments were performed in both the horizontal (XY) and the vertical (X,Y-Z) planes.

3.1. Morphological variation of BNC microstructure across different planes

First, the microstructure of BNC from distinct visual density regions (air-liquid interface, mid-, and deeper regions) was characterized in both planes of BNC (horizontal XY and vertical X,Y-Z) by scanning electron microscopy. The fiber organization (orientation distribution ($^\circ$), dominant direction ($^\circ$) and coherency (%)) was assessed using the plugin *Orientation J* in *Fiji/ImageJ* software. Analyzing the SEM images (Fig. 2), it is possible to observe clear differences in the fiber organization across the orthogonal planes. In the horizontal plane (XY) (Fig. 2a), where the cellulose production occurs preferentially due to the highest oxygen availability, the fiber organization is isotropic, as can be seen in the histograms showing a wide distribution of fiber orientations. Indeed, a significant fraction of the fibers are found in every direction, with dominant directions varying from -11 to -44° and the coherency values approaching zero. Moreover, qualitatively, it is possible to notice a decrease in the density of the fibers from the upper air-liquid interface to the deeper regions, with a gradual transition from thicker fiber ribbons at the surface to thinner ones in the deeper zones. This observed gradient in BNC pellicles cultured under static conditions has been reported by other authors as well [46].

Observing the microstructure from a vertical perspective (growth plane, Z-axis), a transition in fiber organization can be noticed from the upper air-liquid interface region to deeper areas. At the air-liquid interface, the fibers exhibit a more aligned structure with a preferred near-horizontal (XY plane, approaching 0°) dominant direction (79 % of fibers oriented between -20.5 and 20.5°) and higher coherency value (0.31). This suggests more alignment along the plane of the membrane (there's a stronger tendency towards a single color (more bluish-green, indicating angles closer to 0°). With increasing depth, the organization becomes more variable, with a broader orientation distribution and a dominant direction that oscillates away from 0° , with lower coherency. Nevertheless, 48–49 % of fibers are oriented between -20.5 and 20.5° from the middle (inner core) to deeper (bottom) regions. This suggests a transition from an anisotropic, directionally aligned fiber network at the

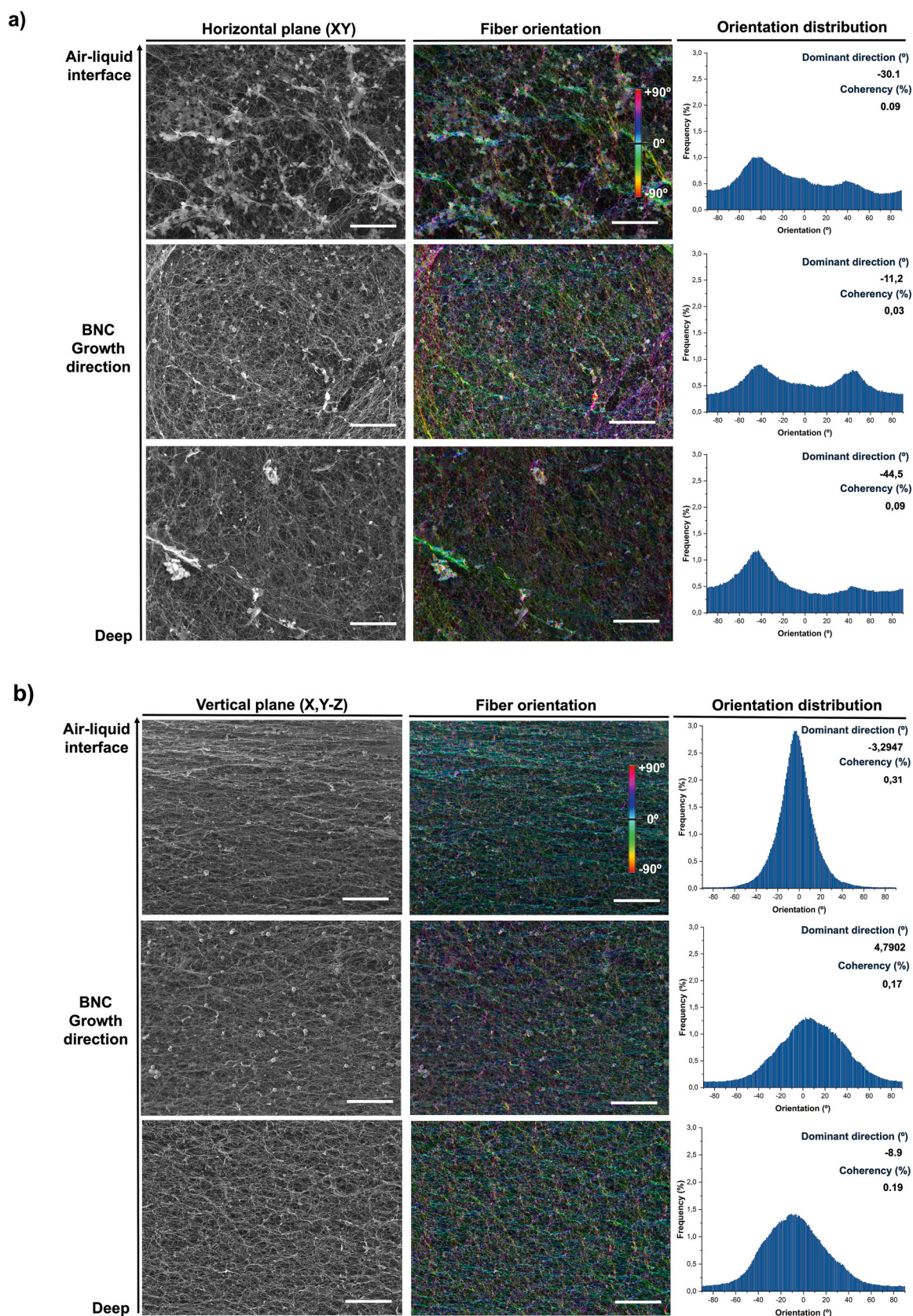


Fig. 2. Representative images of the morphological organization of BNC microstructure from deeper to the superficial region (air-liquid interface). Analysis of fiber orientation, colored fiber map according to angular direction (fibers are colored by angular orientation, as indicated by the color coding bar from -90° to 90°), orientation distribution ($^\circ$), dominant direction, and respective coherency according to different planes of BNC membrane: horizontal (XY) plane (a), and vertical (X, Y-Z) planes (b) (scale bar 10 μm).

surface to a more randomly oriented structure. Despite the increasing tendency towards isotropy with depth, the fibers still show some degree of orientation, favoring the horizontal (XY) direction. In other words, a gradient of anisotropy degree is observed in a vertical Z-axis of the membrane.

In summary, based on image analysis of orthogonal planes observed at different depths, we can state that, under the tested conditions and using this particular strain, statically produced BNC inherently exhibits a variable degree of anisotropy in its 3D microstructure. The horizontal (XY) plane is isotropic, while its vertical (X,Y-Z) plane exhibits a variable anisotropy with an increasing tendency for isotropy with increasing depth. Also noteworthy, unlike some other reports in the literature, we did not see well-separated layers in the images of the vertical sections.

The three-dimensional anisotropy of BNC microstructure can be explained by the cellular dynamics in the static fermentation. In this regime, the BNC production preferentially occurs at the air-liquid interface, where oxygen availability is maximum [47]. Due to the random bacterial movement at the interface, an isotropic BNC mesh is generated in the horizontal (XY) plane. This process continues until saturation of the surface, which leads to a downward movement of the “complete” BNC layer, leaving room for biosynthesis to proceed at the interface. This continuous process results in the sinking of the successively produced isotropic XY layers; when viewed vertically, these layers exhibit a natural preferential horizontal orientation. Considering this mechanism of biosynthesis, we may question to what extent the generated microstructure is interconnected in the Z-direction. This aspect of the structure, as well as the varying isotropic character at different depths, will be discussed below.

3.2. Cross-linking density in different planes

For a deeper understanding of the three-dimensional microstructural organization of the BNC network, the cross-linking density was also investigated in the orthogonal planes XY and X,Y-Z. Considering the greater fiber alignment parallel to the XY plane, associated with a higher packing within this plane, we wondered about the implications of the biosynthesis mechanism with regards to the organization on the Z-direction; possible outcomes could theoretically be: i) well packed layers interspaced with sections bearing lower density of fibers or ii) a continuously filled 3D space without detectable physical separation

between layers. The two scenarios correspond to different degrees of cohesion between the layers. We hypothesized that an answer to this question could be found by comparing the cross-linking density in horizontal (XY) and vertical (X,Y-Z) planes.

Thin BNC pellicles extracted from the visually homogeneous mid-inner core region were used for this analysis. This section was chosen because it is the more representative (in the case of a relatively thick membrane obtained after 24 days of fermentation), the top and bottom sections (corresponding to a small fraction of the membrane) presenting a less homogeneous structure. STED is a super-resolution microscopy technique providing a subdiffraction spatial resolution, enabling the study of subcellular structures at the nanoscale [48]. Despite, to the best of our knowledge, STED has never been reported for BNC microstructure characterization, this advanced microscopy technique, using stimulated emission depletion to eliminate out-of-focus diffraction noise, allows for high-resolution imaging of the intact BNC network, providing a reliable method for capturing and quantitatively assessing fiber junctions across its orthogonal planes. Fiber segmentation analysis of 3D fluorescence data of the fiber structure by SOAX enables the extraction of the 3D coordinates and quantification of cross-linking points, as described in the experimental section (Fig. 3a).

Since preliminary statistical analysis revealed no significant differences among the three groups ($p > 0.05$), data were treated as a single population for subsequent analysis.

Analyzing the pooled STED images obtained, no statistically significant differences in cross-linking points were observed between the orthogonal planes (Fig. 3b). The analysis of the 3D microvolumes is suggestive of cross-linking density uniformity across the planes. This conclusion is consistent with the images shown in Fig. 2, where stratified layers could not be identified across the full Z-axis. Several reports [5,9,24,27,49–51] present images where such layered (stratified) structures can be recognized. In our case, the samples were prepared by directly freezing the BNC in liquid nitrogen, which we believe may better preserve the structure of the membrane (as compared to other freezing techniques). The obtained cross-linking values— 5.8 and 6×10^8 counts/mm³ for horizontal (XY) and vertical (X,Y-Z) planes, respectively—are in the same range of density values reported by Takayama et al. [10] for BNC produced by several strains.

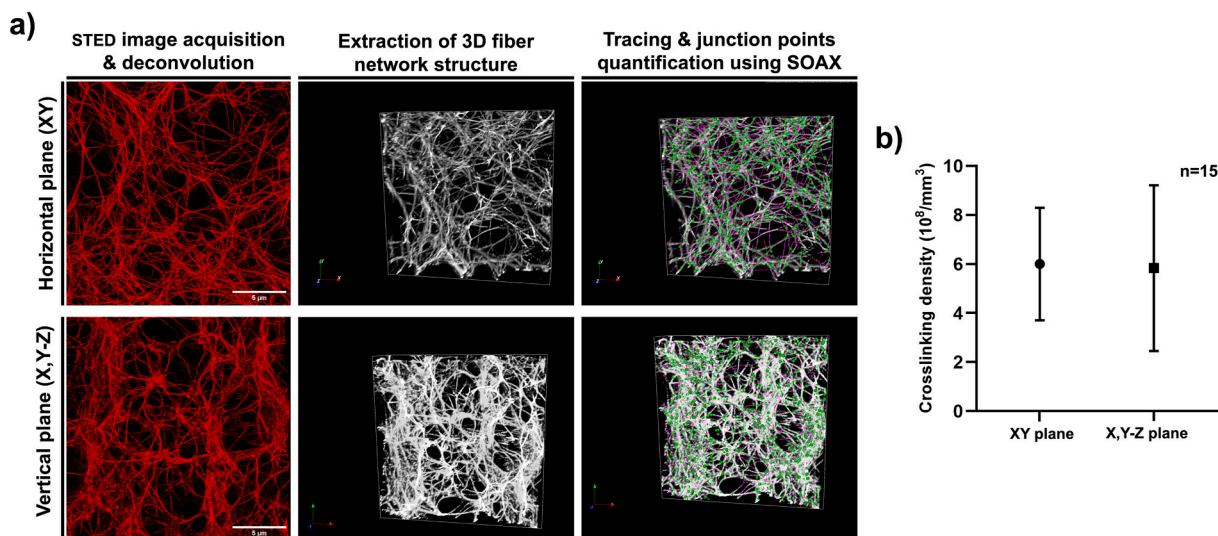


Fig. 3. (a) Overview of the STED image analysis: 3D data collection of fluorescence-stained BNC network using STED, image deconvolution and processing, followed by fiber segmentation (magenta lines) with identification of junction points (green dots) using SOAX software. (b) Crosslinking density for each BNC plane (XY and X,Y-Z). Data are presented as mean values $\pm$ SD. A non-parametric two-tailed Mann-Whitney test was conducted to compare the non-normally distributed data. No statistically significant differences were observed ($U = 75$, $p = 0.6579$) and the rank-biserial correlation was 0.11, indicating a minimal effect size. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. On the generation of the anisotropy gradient

While Gromovkyh et al. [9] have proposed a plausible model to explain the formation of a layered microstructure, no investigations have yet comprehensively examined how static culture influences the native three-dimensional microstructure of BNC and how this correlates with mechanical response. Additionally, those models argue that no density gradients are established under slower production rates, resulting in an isotropic BNC network [9].

This study evidenced that the statically produced BNC membranes exhibited an anisotropic three-dimensional network that gradually dissipates with depth, i.e., an anisotropy gradient was observed on the X,Y-Z planes. Based on the collected experimental evidence, we propose a model, illustrated in Fig. 4, that elucidates the observed anisotropy gradient. In a static regime, BNC production occurs predominantly at the air-liquid interface [47]. A highly entangled isotropic network is formed by permanent cross-linking crystalline junctions and transient contact junctions [10]. Once this initial BNC layer saturates, it sinks into the culture medium, allowing for the continuous production of new BNC layers at the air-liquid interface. This iterative process results in the thickening of the BNC membrane over time. As the initially formed layers sink downward and undergo prolonged submersion in the culture medium, the extended exposure results in greater swelling compared to the more recently synthesized superficial layers. The swelling within the thickening BNC membrane generates a corresponding stretching in the downward direction. This results in fiber re-orientation from near-horizontally aligned fibers, at the air-liquid surface regions, to a more angular arrangement with depth. This swelling-driven effect likely explains the often-reported top-to-bottom decreasing fiber density in BNC pellicles cultured under static conditions [46]. This proposed mechanism is more plausible than variations in bacterial density or nutrient availability, as most BNC synthesis occurs in-plane at the air-liquid interface [10]. The swelling-driven gradient stretching progressively dissipates the initially anisotropic planar fiber network into a more disordered, isotropic structure as the membrane thickens.

Bacterial strain and static culture conditions (e.g., incubation time, medium composition) influencing productivity and consequently fiber density and membrane thickness are expected to affect the degree of 3D anisotropy by modulating swelling-driven gradient stretching along the

Z-direction. Thus, introducing the concept of swelling-driven gradient stretching into the analysis offers a plausible model to explain the diverse BNC microstructures under static culture conditions reported in the literature. Moreover, post-processing operations may be conceived whereby membrane stretching (mechanical, osmotic) may give rise to different degrees of anisotropy, biomechanically relevant.

3.4. Mechanical properties: stress-strain response

BNC samples collected from the horizontal (XY) and vertical (X,Y-Z) planes were submitted to uniaxial tensile forces to analyze the differential mechanical behavior and fiber reorganization. The stress-strain curves of BNC samples obtained are depicted in Fig. 5a. It is possible to notice clear differences comparing the mechanical response of BNC collected from distinct membrane planes (XY and X,Y-Z).

Like many biological tissues such as skin [52], blood vessels [37,53], and ligaments [54], composed of highly ordered collagen forms (triple helix, fibrils, and fibers), wet BNC exhibits a J-shaped stress-strain behavior due to the hierarchical fiber network microstructure. J-shaped curves are often characterized by three distinct stages, the first corresponding to gradual fiber alignment via bending and twisting with negligible stiffness and compliant mechanics (I), followed by an increase in tangent modulus (II). In the third phase, fibers are straightened, and the stress behavior curve is governed by the stretching of the fibers, giving a relatively linear response and high tangent modulus (III) [55]. This J-shaped response was also seen in stress-strain curves of BNC in both orientations, consistent with the reported BNC viscoelastic response with the absence of linear stress-strain behavior at low deformations, being suggestive of network rearrangements [20,22,23,25]. When the horizontal plane (XY) is subjected to tension forces, the initial non-linear response of the stress-strain curve, observed until approximately 20–30 % of strain, corresponds to an initial fiber reorganization and alignment in the direction of the tension (I and II) (Fig. 5a). Afterward, the straightened fibers are recruited to resist the increasing applied deformation with increasing linear stiffness (III), exhibiting a higher strain hardening response. In the case of the vertical planes (X,Y-Z), it can be noticed that the non-linear region of the stress-strain curve, corresponding to fiber rearrangement in the direction of the tension (I and II), occurs for higher strain (%) ranging from 60 to 80 %, and the

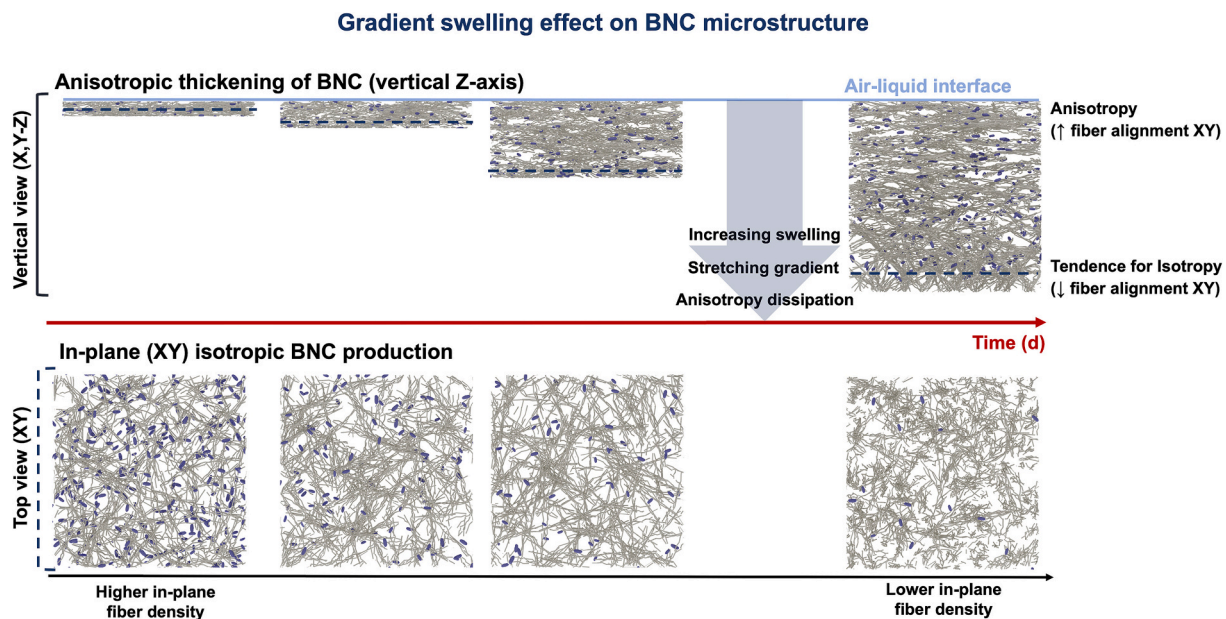


Fig. 4. Scheme illustrating the effect of swelling-induced gradient stretching on the reorganization of BNC microstructure. Top: vertical view (X,Y-Z plane), highlighting the anisotropic thickening of BNC with time and also a higher fiber alignment on air-liquid interface and increasing tendency for isotropy with depth. Bottom: top view (XY plane), highlighting the in-plane isotropic BNC production.

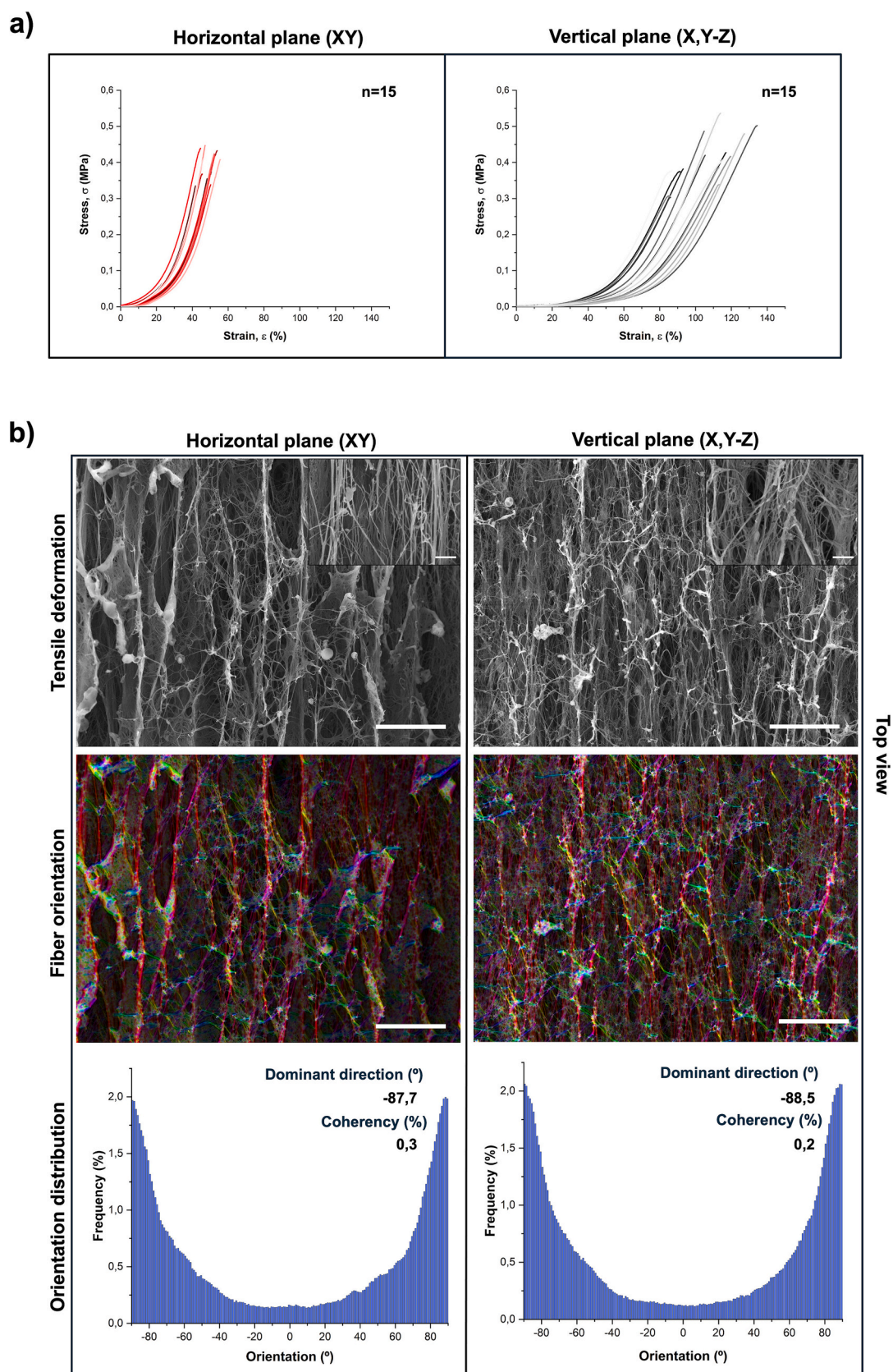


Fig. 5. a) Stress-strain curves of distinct BNC growth planes (horizontal XY and vertical X,Y-Z). b) SEM images of fiber network deformation (scale bar: 10 μm and 1 μm insets) and image analysis after rupture. The applied tensile forces are vertical in both images.

stiffening of the material under tension (III) is significantly lower as compared to the horizontal (XY) plane (Fig. 5a).

As no statistically significant differences were detected between biological replicates ($p > 0.05$) the 15 measurements were pooled and analyzed as single population for each BNC plane. The horizontal (XY) plane exhibited a more rigid and less deformable behavior with a statistically significant two-fold higher Young's modulus (1.9 MPa; **** $p < 0.0001$) and lower elongation at break (Table 2), while the vertical planes (X,Y-Z) exhibited a softer (0.9 MPa) and more deformable response with a 2.2-fold higher elongation at break (107.1 %; **** $p < 0.0001$). However, it is important to note that both BNC planes have a similar ultimate tensile stress (UTS), within the range of 0.39–0.41 MPa (Table 2). The obtained values of tensile mechanical properties for the horizontal plane (XY) are within the range of the previously reported values for ATCC 700178 (Table 2) [21].

3.5. Interplay between microstructure and mechanical properties

The observed anisotropic mechanical response, regarding strain hardening response, is certainly linked to the differences in the microstructural organization of BNC discussed in the previous sections. Remarkably, the similar ultimate tensile strength observed in both planes strongly suggests that the number of connection points between fibers that hold the membrane together (branching and transient contact points, mechanical interlocking) is similar when the membrane is stretched in both directions. Indeed, as observed in Fig. 5b, the membrane stretched in either direction results in a similar morphology, displaying fully stretched fibers.

The preferred horizontal fiber orientation explains the faster and easier fiber co-linearization when BNC is stretched in XY plane, enabling a more efficient load transfer along co-linear fibers with the formation of direct load paths (Fig. 5b, inset horizontal plane), maximizing stiffness and reducing deformation (Fig. 6). Amason et al. [49], reported a similar behavior, demonstrating that in a stratified BNC-gelatin system, the thickness and orientation of the BNC laminae relative to the direction of the applied load significantly influence the mechanical performance. Specifically, thicker and highly oriented BNC laminae in the load direction resulted in higher specific modulus and strength. Also, Gromovikh et al. [9] have demonstrated that anisotropic layered freeze-dried BNC films exhibited higher stiffness and strength and are less deformable when compared to isotropic freeze-dried BNC films. Conversely, when the vertical planes (X,Y-Z) are stretched out, the fibers, predominantly oriented within the XY plane, must align perpendicularly to their intrinsic primary orientation. This involves rotational and bending deformations of the fibers, leading to a more difficult and slow fiber co-linearization and a less efficient load distribution (Fig. 5b, inset vertical planes), resulting in a prolonged initial non-linear response, increased total deformation, and reduced stiffness (Fig. 6). This is responsible for the larger elasticity and softness of BNC when stretched in the Z-direction. Also, the greater stretching ability in the Z-direction compared to the horizontal direction may result from a higher maximum length allowed in this axis before rupture.

Table 2
Mechanical properties of ATCC 700178 in two different orientations (XY plane and X,Y-Z): ultimate tensile stress (MPa), Young's modulus (MPa), and breaking elongation (%). Data are presented as mean values $\pm$ SD. Ultimate tensile stress data were normally distributed with equal variances and were analyzed using an unpaired *t*-test ($p = 0.3126$, not significant; Cohen's $d = -0.38$, indicating a small effect size). Young's modulus and breaking elongation data were normally distributed with unequal variances ($p = 0.0043$ and $p < 0.0001$, respectively) and were analyzed using unpaired *t*-tests with Welch's correction. Both comparisons showed significant differences (**** $p < 0.0001$) with large effect sizes (Cohen's $d = -5.09$ and -5.15 for Young's modulus and breaking elongation, respectively), indicating significant group differences.

ATCC 700178	Ultimate tensile stress (MPa)	Young's modulus (MPa)	Breaking elongation (%)
Vertical (X,Y-Z)	0.41 $\pm$ 0.07	0.97 $\pm$ 0.11	107.1 $\pm$ 15.5****
Horizontal (XY)	0.39 $\pm$ 0.05	1.93 $\pm$ 0.25****	48.8 $\pm$ 3.9

Finally, following distinct tensile mechanical responses leading to a similar degree of final fiber orientation (Fig. 5b), both planes ruptured at similar ultimate tensile stresses (Table 2). A recent study by Takayama et al. [10] reported that the shape of the stress-strain curve, i.e., the strain-hardening response and the rupture strength, is primarily governed by the number of stress transfer paths formed during deformation, which is determined by the cross-link density rather than cellulose content and fiber thickness, which influence the magnitude of the stress [10]. Transposing these findings to the present study, the observed isotropy in cohesive forces across orthogonal planes, as indicated by similar cross-linking density and ultimate tensile stresses, suggests that the anisotropic strain-hardening response likely results from a distinct spatial arrangement of these stress-transfer points across planes with respect to the applied tensile force direction, influencing the efficiency of loading distributions during stretching, as previously discussed. Accordingly, the isotropy in the cohesive forces observed for both planes likely dictates the similar ultimate tensile stress, which is not directional dependent, in this case, but rather a result of similar cumulative cohesive network interactions—from fiber ramification to van der Waals bonding forces and mechanical interlocking, and intrinsic properties of BNC individual fibers (crystallinity, degree of polymerization, fiber thickness).

The BNC's mechanical anisotropy has long been recognized in the literature, due to the high tensile modulus along the native fibril direction and low compressive modulus in the perpendicular direction [56]. Few studies on BNC anisotropy have focused on the comparison of mechanical behavior across orthogonal orientations. Nevertheless, the directional dependency of mechanical properties of BNC and BNC-based double networks in compression and stress-relaxation studies [57,58] and tensile response [59] has been investigated. Pogorelova et al. [59] reported minimal differences in the mechanical properties of BNC, including Young's modulus, ultimate tensile stress, and breaking strain, under tensile loading in orthogonal directions, with a low anisotropy coefficient of 1.309. However, our integrated and complementary characterization approach across BNC orthogonal planes suggests that static production results in an inherent and variable degree of anisotropy of BNC microstructural organization, owing to the biosynthesis mechanism at the air-liquid interface and swelling-induced stretching. This 3D microstructural anisotropy generates an anisotropic strain hardening response. We speculate that more effective stretching is predicted to produce a more homogenous fiber arrangement in both the horizontal (XY) and vertical (X,Y-Z) directions, reducing differences in fiber rearrangement during tensile deformation and therefore reducing the mechanical anisotropy.

3.6. Constitutive law identification and validation: HGO model

Evolutionary algorithms (EA) have demonstrated effectiveness in the iterative optimization of finite element (FE) model updating processes. In the framework of the identification of the constitutive laws, this approach involves defining an objective function based on differences between nodal displacements (FEA) observed in experimental and

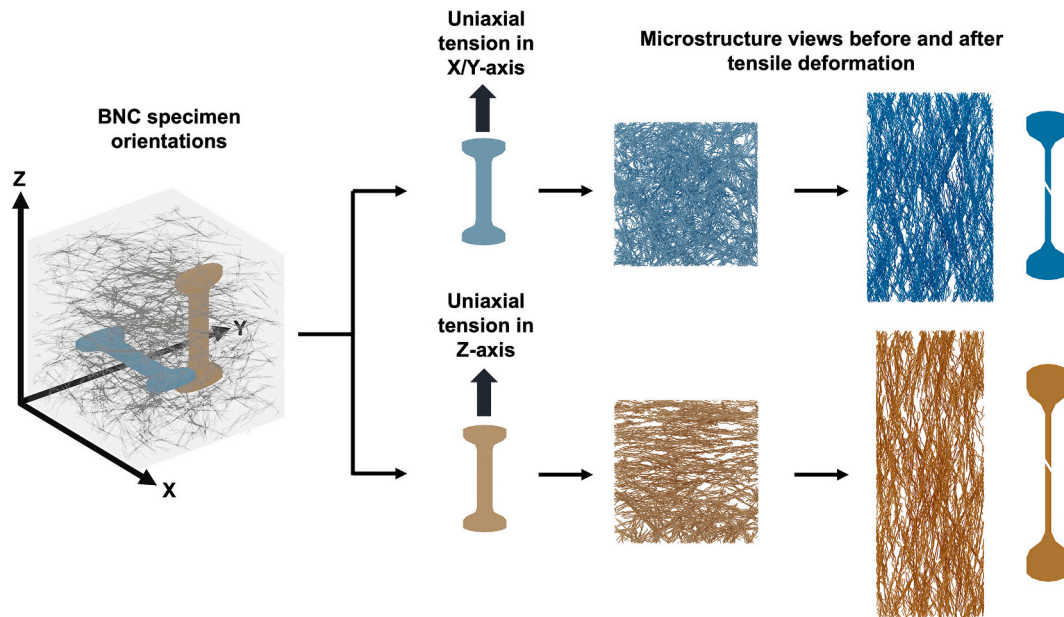


Fig. 6. A schematic representation of the tensile mechanical tests performed over different BNC growth planes. Dog-bone BNC specimen orientations (XY and X,Y-Z) and illustrative representation of the microstructure across distinct planes before and after uniaxial tension, until rupture.

numerical datasets. A notable advantage of EA lies in its reduced dependency on initial solutions, minimizing the risk of convergence to local minima during optimization [36,39,60–63].

The Holzapfel-Gasser-Ogden (HGO) model was found to provide an effective approximation of the anisotropic mechanical response within the range of quasi-static deformations typically observed in BNC experimental results. The application of the Inverse Method (IM) allowed us to identify a set of material HGO law coefficients (Table 1 of the supplementary material) based on average true stress-strain curves obtained experimentally for each BNC specimen orientation (Fig. 7). Differences in HGO coefficients were noticed for the horizontal (XY) and vertical (X,Y-Z) orientations of BNC. Specimens harvested along the vertical directions exhibited higher isotropic stiffness ($C_{10} = 0.002382$ MPa) compared to the horizontal one ($C_{10} = 0.001165$ MPa). This may be due to higher water release in the course of the loading process along the vertical direction being responsible for a higher value of shear modulus G . In agreement with this behavior, the compressibility D in vertical directions is significantly higher ($D = 2.716860$) than in the horizontal one ($D = 0.086090$). This reflects a greater predisposition to volumetric deformation in vertical directions, which is in line with the

higher breaking elongation (%) exhibited for this plane, as represented in Table 2. The coefficients associated with fiber stiffness and material nonlinearity, k_1 and k_2 , respectively, allow us to define the anisotropic behavior of BNC under tensile loading. In vertical directions, those coefficients ($k_1 = 0.039461$ and $k_2 = 0.118547$) are inferior to the ones obtained in the horizontal direction ($k_1 = 1.675240$, $k_2 = 2.890240$). The higher value of k_1 in the horizontal direction is directly related to the greater tensile stiffness of the fibers, which respond more quickly when loaded. The coefficient k_2 behaves as a geometric parameter independent of the initial fiber orientation. These values suggest that the BNC mechanical response is predominantly influenced by fiber alignment along the XY plane, offering greater resistance to tensile loading. This behavior follows the preferential alignment of BNC fibers during the biosynthesis process [38], which is discussed and depicted in Fig. 4. The greater dispersion of the fibers in vertical (X,Y-Z) planes relative to the loading direction, represented by the higher value of κ , is associated with a lower contribution of fiber stiffness k_1 in this direction. Applications requiring high levels of compliance, like adhesives or biological membranes, may benefit from the improved deformability observed in the transverse direction [38].

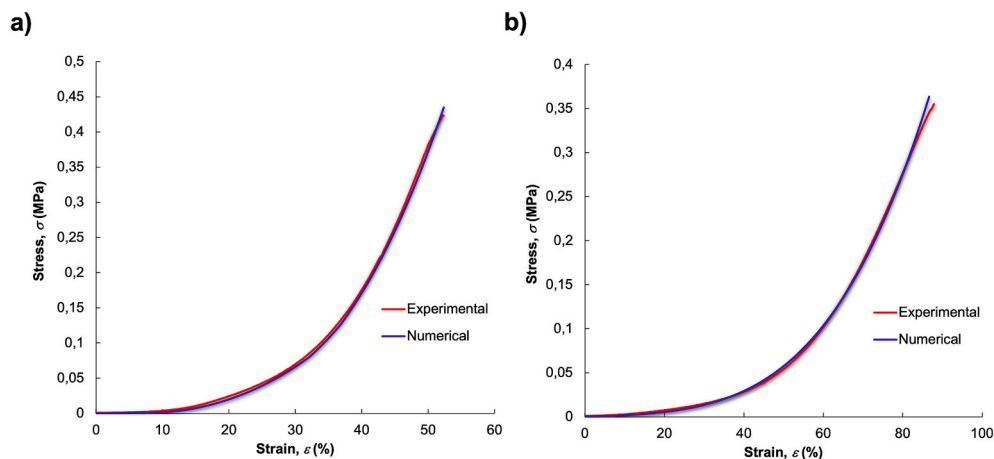


Fig. 7. Stress-strain numerical agreement for: (a) horizontal and (b) vertical loading, putting in evidence the agreement between experimental and numerical data based on evolutionary algorithms.

The adopted methodology, based on EA, proved to be highly effective in reproducing the experimental data (stress-strain curves in Fig. 7) in both directions. The ability of EA to explore the solution space effectively was particularly advantageous in this study, given the highly non-linear and multidimensional nature of the parameters' optimization process. More specifically, the results demonstrated that the HGO model was exceptionally accurate in replicating the anisotropic mechanical behavior of BNC under quasi-static tensile loading. The attained numerical agreement in both horizontal and vertical directions (Fig. 7) emphasizes the suitability of the HGO model for materials with directionally dependent mechanical responses.

The validation process, which involved finite element (FE) analysis under tensile loading, further confirmed the reliability of the calibrated material law. The close alignment between FE predictions and experimental data for both loading directions (Fig. 7) highlights the accuracy and predictive capability of the identified hyperelastic coefficients. The high reliability of the FE simulations (Fig. 8) reinforces the robustness of the combined numerical-experimental methodology. This approach enabled replicating the experimental results in two different material orientations.

These HGO coefficients were used as input in an independent FE analysis to verify the accuracy of the developed inverse strategy to replicate the obtained experimental data accurately. A 3D finite element model was developed, consisting of 3321 six-node reduced-integration shell elements (S6R). The element size was refined through a mesh convergence study to ensure the accuracy while maintaining a reasonable computational cost. Boundary conditions replicated the uniaxial tension test setup (Fig. 8a): the bottom nodes of the sample were fully constrained, while a displacement was applied to the nodes situated along the opposite edge in the loading direction. In this scenario, symmetry conditions were not used to reproduce the full experimental setup. The model was solved using a static general step following a nonlinear geometry analysis. In this sense, an FE model of the tested specimen was developed (3321 six-node shell elements), with boundary conditions matching the uniaxial tension test carried out experimentally (Fig. 8a). Numerical results showed a satisfactory agreement between FE predictions and experimental stress-strain curves for both loading directions (Fig. 8b).

This approach enabled replicating the experimental results in two different material orientations and provided insights into the fundamental mechanisms governing the anisotropic mechanical response of static cultured BNC. Nonetheless, while the HGO model provides an excellent approximation of the quasi-static behavior of BNC, its applicability under dynamic or multiaxial loading scenarios remains untested in this study. However, given that many BNC-based applications involve predominantly planar loading modes, the current validation under in-

plane uniaxial loading conditions offers a meaningful and relevant representation of typical functional performance. This limitation highlights the need for further investigations to assess the model's predictive capabilities under more complex loading conditions, which are often encountered in practical applications. While the results are promising, future research should explore the viscoelastic behavior of BNC under dynamic loading conditions. Also, investigating thermal or environmental dependencies of material behavior could further expand the applicability of the HGO model in real-world scenarios.

Furthermore, the integration of the HGO constitutive model with an inverse parameter identification strategy based on evolutionary algorithms, as applied in this study, establishes a validated and generalizable framework for the mechanical characterization of anisotropic materials. Owing to its strong theoretical foundation and capability to capture direction-dependent hyperelastic behavior, the HGO model is especially suited for modeling fiber-reinforced soft tissues, bioengineered scaffolds, and other anisotropic biomaterials. Thus, the methodology developed here, which combines robust experimental data, inverse modeling, and finite element analysis, can be easily extended to other material systems that display anisotropic mechanical responses, thereby contributing to the predictive design and optimization of advanced biomimetic structures.

Although the hyperelastic formulation employed in this study adequately reproduced the mechanical response of BNC under quasi-static uniaxial loading, it does not capture potential time-dependent (viscoelastic) effects. The decision to neglect viscoelasticity was based on the short duration of the mechanical tests and the absence of observable rate-dependent phenomena within the test window. Nevertheless, BNC is known to exhibit viscoelastic behavior under specific conditions, including sustained or cyclic loading. Therefore, future investigations should incorporate dynamic mechanical analysis, such as creep, stress-relaxation, or frequency-dependent testing (DMA), to assess the viscoelastic properties of BNC and to refine the constitutive framework accordingly, potentially through visco-hyperelastic formulations.

4. Conclusions

Bacterial nanocellulose, with its intricate nanofibrillar network resembling extracellular matrix (ECM) collagen fibers, is a versatile biopolymer known for its outstanding properties, offering valuable opportunities for advanced biomedical applications.

The present study contributes to a more comprehensive understanding of the microstructural organization of static cultured BNC membranes and their mechanical properties. The fibers are preferentially aligned horizontally in the more superficial layers, but probably as

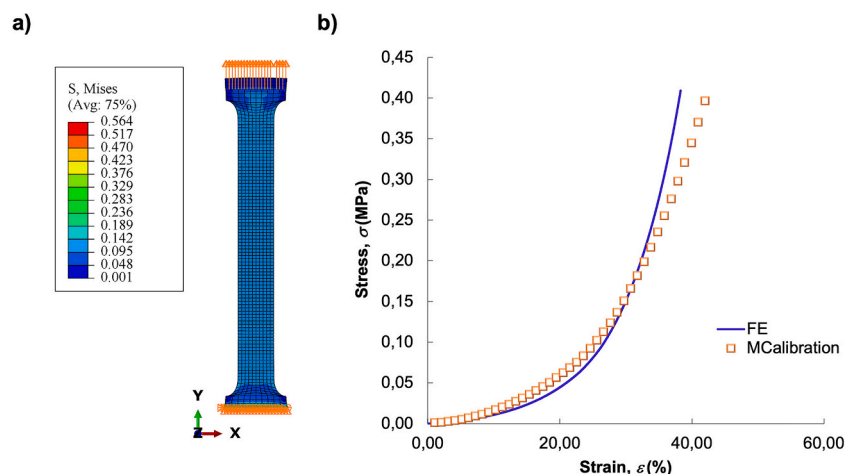


Fig. 8. Finite Element model showing the used boundary conditions (a) and the stress-strain agreement of FE and MCalibration (b).

a result of a swelling-induced fiber reorganization, in deeper layers this organization is partially lost. This preferential horizontal orientation of the fibers generates an anisotropic strain hardening mechanical response, highlighted by a stiffer and less deformable tensile response in the horizontal (XY) plane and a lower stiffness and greater deformability in vertical orientations. Indeed, the anisotropic tensile mechanical response in both planes was corroborated by numerical modeling using finite element analysis. Although the 3D microstructural anisotropy of BNC governs the differential strain hardening mechanical response in different planes, the cohesive forces of the fibrous network appear to be non-directionally dependent, as indicated by the similar ultimate tensile stress and the number of connection points for both planes. Future studies comparing the mechanical response across orthogonal planes of statically produced BNCs with varying degrees of microstructural anisotropy and crosslinking densities may help clarify whether cohesive forces are inherently non-directional and primarily influenced by intrinsic fiber properties, such as crystallinity, degree of polymerization and fiber thickness. In addition, we speculate that mechanical interlocking resulting from fiber organization in the 3D plays a key role in the mechanical properties of the BNC membranes. Moreover, the study of viscoelastic behavior of BNC planes under complex loading and dynamic conditions, as well as the influence of thermal and environmental factors on their mechanical behavior, is envisioned.

Nevertheless, this study provides valuable insights into the anisotropy of static cultured BNC at a structure-property level, contributing to informed BNC production and target-oriented application, expanding the potential applications of BNC. In the biomedical field, BNC's versatility allows it to be tailored to meet the mechanical demands of various tissues in an orientation-dependent way. For instance, its horizontal (XY plane) microstructure suits the development of tissues demanding higher stiffening behavior under tension, such as tendons and ligaments [64]. In contrast, its vertical plane features are better suited for applications requiring greater elasticity and deformability, such as blood vessels [65].

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijbiomac.2025.148114>.

CRediT authorship contribution statement

Lúgia Costa: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Alexandre F. Carvalho:** Writing – review & editing, Methodology, Formal analysis. **António J.S. Fernandes:** Methodology. **Teresa Campos:** Methodology, Investigation. **Nuno Dourado:** Writing – review & editing, Validation, Methodology. **Ana Cristina Rodrigues:** Methodology. **Paula Sampaio:** Writing – review & editing, Methodology. **Florinda M. Costa:** Writing – review & editing, Validation, Supervision. **Fernando Dourado:** Writing – review & editing, Validation. **Miguel Gama:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the strategic fundings: UIDB/04469/2020 unit, by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems (LA/P/0029/2020) and i3N - Institute of Nanostructures, Nanomodelling and Nanofabrication (LA/P/0037/2020, UIDB/50025/2020 and UIDP/50025/2020). L. Costa gratefully acknowledges the Portuguese Foundation for Science and Technology (FCT) for the granted scholarship (2020.05233.

BD).

The authors acknowledge the support of i3S Scientific Platform Advanced Light Microscopy, member of the national infrastructure PPBI-Portuguese Platform of BioImaging.

This research was funded in whole by the Fundação para a Ciência e a Tecnologia, I.P. (FCT, <https://ror.org/00snfq58>) under Grant (2020.05233.BD, UIDB/04469/2020, LA/P/0029/2020, UIDB/50025/2020 and UIDP/50025/2020). For the purpose of Open Access, the author has applied a CC-BY public copyright license to any Author's Accepted Manuscript (AAM) version arising from this submission.

Data availability

The data supporting this study are available along with the article and its supplementary data files are available from the authors upon request.

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