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1 **NANO-/MICROSTRUCTURE OF EXTRUDED SPIRULINA/STARCH FOAMS IN**  
2 **RELATION TO THEIR TEXTURAL PROPERTIES**

3  
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20 **Abstract**

21 This work reports on an in-depth characterization of the nano- and microstructure of  
22 extruded starch foams loaded with the microalga *Spirulina* (1, 5 and 10 wt.%), as well as the  
23 implications of *Spirulina* incorporation on the textural properties of the foams. Due to the  
24 gelatinization process occurring during extrusion, the crystalline and lamellar structures  
25 originally present in the starch granule were disrupted, resulting in very amorphous foams.  
26 Moreover, the crystalline structure of the fatty acids present in the raw microalga was lost  
27 during processing. The presence of *Spirulina* intracellular components induced the formation  
28 of more thermally-stable V-type crystallites through complexation with amylose, hence  
29 producing slightly more crystalline foams ( $X_c \sim 5-9\%$ ) than the pure extruded starch ( $X_c$   
30  $\sim 3\%$ ). This affected the microstructure of the hybrid foams, which showed more densely  
31 packed and well-connected porous structures. Microstructural changes had an impact on the  
32 texture of the foams, which became harder with greater *Spirulina* loadings. The foams  
33 underwent very limited re-crystallization upon storage, which was further reduced by the  
34 presence of *Spirulina*. Interestingly, the free fatty acids from *Spirulina* re-crystallized and  
35 the resistant starch content in the 10% *Spirulina* foam increased, which could potentially be  
36 interesting from a nutritional perspective. These results show the potential of extrusion  
37 cooking to produce healthier snack foods and highlight the suitability of advanced  
38 characterization tools such as neutron tomography and small angle X-ray scattering to  
39 investigate food structure.

40

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42

43 **Keywords:** microalgae; SAXS; WAXS; neutron tomography; gelatinization

44

## 45 **1. Introduction**

46 Extrusion cooking is one of the processing methods used in the food industry to produce  
47 healthier snack foods, which is increasingly being used to replace conventional technologies  
48 such as frying. The process is based on the application of high temperature, pressure and  
49 shear stress, resulting in major structural changes (such as starch gelatinization, protein  
50 denaturation, formation of amylose-lipid complexes and degradation of bioactive  
51 substances) in the raw materials added to the extruder (Camire, Camire, & Krumhar, 1990).  
52 After the cooking step, the melt fluid is shaped into the desired form in the die, thus yielding  
53 the final product. The properties of the manufactured products can be adjusted by modifying  
54 the processing parameters (such as feed moisture, temperature profile, screw speed and feed  
55 rate) (Ding, Ainsworth, Plunkett, Tucker, & Marson, 2006; Ding, Ainsworth, Tucker, &  
56 Marson, 2005; Hashimoto & Grossmann, 2003) and by selecting suitable raw materials.  
57 Cereal flours, rich in starch, are typically the main component in the formulations used to  
58 produce extruded snacks, since they present good processability and enable the production  
59 of snacks with acceptable physical characteristics, i.e. an adequate expansion coefficient,  
60 hardness and density (Guy, 2001; Launay & Lisch, 1983). However, they present low  
61 nutritional value due to their relatively limited protein content and deficiency in certain  
62 essential amino acids (Bressani, Wilson, Béhar, & Scrimshaw, 1960; Howe, Jansen, &  
63 Gilfillan, 1965). In order to improve their nutritional profile, starch-based snack foods have  
64 been enriched with several protein-rich ingredients such as legumes and dairy (Anton, Gary  
65 Fulcher, & Arntfield, 2009; Onwulata, Konstance, Smith, & Holsinger, 2001; Rampersad,  
66 Badrie, & Comissiong, 2003; Veronica, Olusola, & Adebowale, 2006). However, the  
67 incorporation of these additional ingredients into the extrusion formulations does not only  
68 have an impact from the nutritional perspective, but also has technological implications,  
69 since the processing parameters need to be adjusted and the structural, textural and sensory

70 properties of the extruded snacks may be affected (Lazou & Krokida, 2010; Pastor-Cavada,  
71 et al., 2011).

72  
73 Besides demanding products with added nutritional value and health benefits, consumers  
74 are increasingly searching for more natural ingredients, such as algae-based products. In  
75 this context, microalgae are gaining a great interest in the food industry for their use as  
76 nutritional supplements and food additives (Chacón-Lee & González-Mariño, 2010;  
77 Vigani, et al., 2015). Some microalgae species have been used in human nutrition for many  
78 centuries and the interest lays in their high protein content, lipid profile, with relatively high  
79 amounts of  $\omega$ 3 and  $\omega$ 6 fatty acids, and the presence of bioactive compounds such as  
80 carotenoids, phycobilins, polysaccharides, vitamins, and sterols (Chacón-Lee, et al., 2010;  
81 Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). In particular, *Spirulina*, filamentous  
82 blue-green microalgae or cyanobacteria, are one of the most widely used species in the  
83 healthy-food market due to their large content in proteins,  $\gamma$ -linolenic acid and other  
84 compounds such as vitamins (mainly B vitamins), minerals, pigments (phycocyanin,  $\beta$ -  
85 carotene and xanthophyll),  $\gamma$ -tocopherol and phenolic compounds, which confer these  
86 microalgae high antioxidant activities (Khan, Bhadouria, & Bisen, 2005; Miranda, Cintra,  
87 Barros, & Mancini-Filho, 1998). Since these microalgae have received the GRAS  
88 (Generally Recognized As Safe) certification, they have already been incorporated into  
89 different types of food products, such as shakes for elderly (Santos, Freitas, Moreira,  
90 Zanfonato, & Costa, 2016), pasta (De Marco, Steffolani, Martínez, & León, 2014; Fradique,  
91 et al., 2010; Özyurt, et al., 2015) and extruded snacks (Joshi, Bera, & Panesar, 2014;  
92 Bárbara Franco Lucas, de Morais, Santos, & Costa, 2018; Barbara Franco Lucas, Morais,  
93 Santos, & Costa, 2017; Morsy, Sharoba, & Hem, 2014). Although most of the existing  
94 literature has focused on the nutritional aspects, the incorporation of *Spirulina* may have an

95 impact on the structural changes undergone by food during processing. This may be  
96 especially important in the case of starchy foods, whose processing is based on the  
97 occurrence of a phenomenon known as gelatinization. Native starch granules display a  
98 hierarchical structure consisting of alternating amorphous and semi-crystalline growth rings  
99 of 120–400 nm thickness. At the same time, the semi-crystalline rings are formed by a  
100 lamellar structure of alternating crystalline and amorphous regions with a repeat distance  
101 of 9–10 nm (Blazek & Gilbert, 2011). When processed at high temperature and moisture  
102 conditions, starch is known to undergo a gelatinization process in which its hierarchical  
103 structure is disrupted. The gelatinization process is initiated by the access of water towards  
104 the interior of starch granules, which swell and absorb water, followed by the disruption of  
105 the crystalline and lamellar structure and the leakage of amylose from the starch granules  
106 to form a continuous gel. In fact, it has been recently demonstrated that *Spirulina*  
107 microalgae interfere with the gelatinization process of starch and as a result, lipid-amylose  
108 complexes can be formed, promoting the crystallisation of V-type structures (Martínez-  
109 Sanz, Fabra, G. Gómez-Mascaraque, & López-Rubio, 2018). Thus, the incorporation of  
110 *Spirulina* into starch-based snack foods is not only expected to impact the nutritional value,  
111 but also to affect the structural properties of the obtained products. In this study we have  
112 performed a detailed investigation of the structural effects of the addition of *Spirulina* into  
113 corn starch foams produced by means of extrusion cooking. For the first time a combination  
114 of advanced characterisation techniques (ranging from the nanometre to micrometre length  
115 scale), such as X-ray scattering (SAXS/WAXS) and neutron tomography, together with  
116 scanning electron microscopy (SEM), were used to understand the structural changes  
117 occurring in extruded starch foams as result of the addition of *Spirulina* microalgae. Some  
118 of the main advantages of using scattering and tomography techniques are related to their  
119 non-destructive character and their ability able to provide structural information on native

120 samples, since they minimise experimental artefacts derived from sample treatment.  
121 Furthermore, the use of neutrons is particularly interesting for the analysis of food systems  
122 by means tomography due to the higher contrast of neutrons with hydrogens (abundant in  
123 food components), as compared with X-rays. These results are highly relevant in the  
124 development of new food products, since structural changes are expected to affect the  
125 sensory properties and digestibility of the produced snacks.

126

## 127 **2. Materials and methods**

### 128 **2.1 Materials**

129 Commercial corn starch flour (19% amylose) was purchased locally (Sweden). *Spirulina*  
130 *platensis*, in the form of dry powder, was kindly donated by Dr. Acién from the University  
131 of Almeria (Spain). The composition of the raw *Spirulina* in terms of total carbohydrate,  
132 protein, ash, lipids and polyphenols was determined using standard methods. The total  
133 carbohydrate content was determined after sulphuric acid hydrolysis, following the method  
134 described in previous work (Martínez-Sanz, et al., 2019). The protein content was  
135 determined by the Kjeldahl method, using a factor of 6.25 to convert the nitrogen to protein  
136 content (Boussiba & Richmond, 1979). The ash content was determined by the standard  
137 method TAPPI T211 om-07. Lipids were estimated by performing a Soxhlet extraction of  
138 ca. 1g of dry *Spirulina* with 800 ml of toluene:ethanol 2:1 (v/v) for 24 h. The total phenolic  
139 content was estimated by the Folin-Ciocalteau colorimetric assay (Singleton, Orthofer, &  
140 Lamuela-Raventós, 1999). The gross chemical composition of the microalgal biomass was  
141 determined as: 40.6% ash, 26.7% protein, 15.1% lipid; 8.0% carbohydrate and 0.9%  
142 polyphenols.

143

### 144 **2.2 Preparation of starch and starch-*Spirulina* foams**

145 Pure starch and hybrid foams with three different loadings of *Spirulina* (1, 5 and 10 wt.%  
146 dry weight) were prepared by extrusion cooking. The samples were processed with 30 wt.%  
147 moisture content to facilitate starch gelatinization during processing and let to equilibrate for  
148 24 hours before extrusion. A single-screw extruder (Brabender Duisburg DCE-330,  
149 Germany) equipped with a variable speed D-C drive unit was used. The extruder barrel had  
150 a 25 mm diameter with length to diameter ratio (L: D) of 20:1. The screw was a 2:1  
151 compression ratio with a linearly tapered rod and 20 equidistantly positioned flights. A round  
152 circular die with a 2 mm diameter and a length of 90 mm was used. Extrusion temperature  
153 was kept constant at 120 °C, 150 °C, 150 °C and 140 °C for zones 1, 2, 3 and 4 respectively.  
154 A screw speed of 150 rpm was used for all extrusion processes. The extrudates were  
155 collected when the operation conditions were at constant steady state. Each formulation was  
156 processed twice. The obtained samples were stored in an equilibrated relative humidity  
157 cabinet at 30% RH and 14 °C for two-three days (T0) and one month (TF) prior to their  
158 characterization. Expansion index was calculated as the ratio of the diameter of the extruded  
159 sample to the diameter of the die.

160

### 161 **2.3 Attenuated total reflectance (ATR) FT-IR analysis**

162 Small pieces from the produced foams were grounded into powder and the obtained  
163 materials were analysed by FT-IR in attenuated total reflectance (ATR) mode using a  
164 Thermo Nicolet Nexus (GMI, USA) equipment. The spectra were taken at 4 cm<sup>-1</sup> resolution  
165 in a wavelength range between 400-4000 cm<sup>-1</sup> and averaging a minimum of 32 scans.

166

### 167 **2.4 Determination of resistant starch content**

168 Resistant starch content was measured using the Megazyme Resistant Starch Assay Kit  
169 (Megazyme International, Wicklow, Ireland), based on the AACC method 32-40.01 (AACC,



170 2009). Extruded samples were grounded to a fine powder in a blender. The samples (100  
171 mg) were incubated with 4 mL mixture of pancreatic  $\alpha$ -amylase and amyloglucosidase for  
172 16 h (at 37 °C, 200 strokes/min) in a shaking water bath (Julabo, Seelbach, Germany) to  
173 hydrolyze digestible starch to glucose. The reaction was terminated with 4 mL of 99 %  
174 ethanol and the indigested resistant starch fraction was obtained after centrifugation (1500  
175  $\times$ g, 10 min). The residue was washed twice with 50 % ethanol and solubilized with 2 mL of  
176 KOH solution 2 M in an ice bath. The solution was neutralized after 20 min with 8 mL  
177 sodium acetate buffer (1.2 M, pH 3.8), and incubated with amyloglucosidase (0.1 mL, 3300  
178 U/mL) at 50 °C for 30 min. Aliquots (0.1 mL) of the diluent were taken and mixed with 3  
179 mL of glucose oxidase/oxidase reagent (GOPOD) and the mixture was incubated at 50°C  
180 for 20 min. Absorbance at 510 nm was measured using a spectrophotometer (Ultrospec,  
181 Biochrom, Cambridge, Germany). The samples were measured in duplicates and two  
182 different batches of extruded samples were assessed. Resistant starch was calculated as g per  
183 100 g of dry sample.

184

## 185 **2.5 Scanning electron microscopy (SEM)**

186 SEM measurements were conducted on a Hitachi microscope (Hitachi S-4800) at an  
187 accelerating voltage of 10 kV and a working distance of 8-16 mm. The pure and composite  
188 starch films were cryo-fractured after immersion in liquid nitrogen to observe the cross-  
189 sections. The samples were then sputtered with a gold-palladium mixture under vacuum  
190 during 3 minutes before their morphology was examined.

191

## 192 **2.6 Simultaneous small and wide angle X-ray scattering (SAXS/WAXS) experiments**

193 Combined small and wide angle X-ray scattering (SAXS and WAXS, respectively)  
194 experiments were carried out in the Non Crystalline Diffraction beamline, BL-11, at the

195 ALBA synchrotron light source ("ALBA synchrotron"). The obtained foams were  
 196 characterized shortly after being processed ( $t_0$ ) and after prolonged storage at 30% RH ( $t_f$ ),  
 197 both in their native dry state and after being hydrated in excess water for at least 24 h. The  
 198 energy of the incident photons was 12.4 KeV (an equivalent wavelength,  $\lambda$ , of 1 Å). The  
 199 SAXS diffraction patterns were collected by means of a single-photon counting detector,  
 200 Pilatus 1 M, with an active area of 168.7×179.4mm<sup>2</sup>, an effective pixel size of 172×172 μm<sup>2</sup>  
 201 and a dynamic range of 20 bits. The sample-to-detector distance was set to 7740 mm,  
 202 resulting in a q range with a maximum value of  $q=0.189 \text{ \AA}^{-1}$ . The WAXS diffraction patterns  
 203 were collected by means of a 3 CCD detectors, Rayonix LX255-HS, with an active area of  
 204 85×255mm<sup>2</sup>, an effective pixel size of 44×44 μm<sup>2</sup> and a dynamic range of 16 bits. In this  
 205 case, the sample-to-detector distance was set to 144.3 mm, corresponding to a maximum q  
 206 value of 8.023 Å<sup>-1</sup>. This detector was tilted with a pitch of 29.38°. The exposure time,  
 207 common to both detectors, was 2 s. This time was optimized in preliminary measurements,  
 208 in order to maximize the signal to noise ratio while avoiding detector saturation.

## 209 2.7 SAXS/WAXS data fitting

210 SAXS data were fitted using the Igor NIST analysis macro suite (Kline, 2006) and applying  
 211 a mathematical function consisting of a power-law term plus one Gaussian-Lorentzian  
 212 peak, similar to that previously reported for several starch samples (A. Lopez-Rubio, et al.,  
 213 2007; Salman, et al., 2009):

$$\begin{aligned}
 214 \quad I(q) = & A \cdot q^{-m} + \left[ R \cdot \left[ I_{max} \cdot \left( 1 + \left( \frac{2 \cdot (q - q_{max})}{\Delta q} \right)^2 \right)^{-1} \right] + \left[ (1 - R) \cdot \left[ I_{max} \cdot \exp \left[ -\frac{1}{2} \cdot \right. \right. \right. \right. \\
 215 \quad & \left. \left. \left. \left( \frac{q - q_{max}}{\Delta q} \right)^2 \right] \right] \right] + bkg \quad (1)
 \end{aligned}$$

216 The first term in equation (1) corresponds to the power-law function (where  $A$  is a prefactor  
217 and  $m$  is the power-law exponent) to account for the underlying diffuse scattering, the  
218 second and third terms correspond to the Lorentzian and Gaussian functions used to  
219 describe the starch lamellar peak (where  $q_{max}$  is the peak position,  $I_{max}$  is the intensity of  
220 the peak,  $\Delta q$  is the full width at half maximum and  $R$  is the Lorentzian to Gaussian ratio of  
221 the peak shape) and the fourth term accounts for the incoherent background signal.

222

223 WAXS peak fitting was performed in Igor, following the procedure described in a previous  
224 work (Amparo Lopez-Rubio, Flanagan, Gilbert, & Gidley, 2008). The obtained values  
225 from the fitting coefficients are those that minimize the value of Chi-squared, which is  
226 defined as:

$$227 \chi^2 = \sum \left( \frac{y - y_i}{\sigma_i} \right)^2 \quad (2)$$

228 where  $y$  is a fitted value for a given point,  $y_i$  is the measured data value for the point and  
229  $\sigma_i$  is an estimate of the standard deviation for  $y_i$ . The curve fitting operation is carried out  
230 iteratively and for each iteration, the fitting coefficients are refined to minimize  $\chi^2$ . The  
231 crystallinity index  $X_C$  was determined from the obtained fitting results by applying the  
232 following equation:

$$233 X_C(\%) = \frac{\sum A_{Crystal}}{A_{Total}} \times 100 \quad (3)$$

234 where  $A_{Total}$  is the sum of the areas under all the diffraction peaks and  $\sum A_{Crystal}$  is the sum of  
235 the areas corresponding to the crystalline peaks.

236

## 237 **2.8 Neutron tomography**

### 238 **2.8.1 Data acquisition**

239 Neutron tomography experiments were performed at the cold neutron imaging station,  
240 IMAGINE (Ott, Loupiac, Désert, Hélyary, & Lavie, 2015) at the ORPHEE reactor of the  
241 French national neutron facility, the *Léon Brillouin Laboratory* (Saclay, France). The 4 types  
242 of foams produced by extrusion were scanned: pure starch (n=2), 1% *Spirulina* (n=3), 5%  
243 *Spirulina* (n=3) and 10% *Spirulina* (n=3), where n equals the number of scanned samples.  
244 The scanning parameters were the following: exposure time, 60s and number of projections,  
245 180; number of dark and flat-field projections, 10; sample-to-detector distance, 50 mm; field  
246 of view (FOV), 47mm × 40mm; effective isotropic pixel size, 18.5 μm. The characteristics  
247 of the utilized detector setup were the following; a Neo sCMOS (ANDOR) camera equipped  
248 with a sCMOS sensor (effective number of pixels, 2560 x 2160 pixels<sup>2</sup>; physical pixel size:  
249 6.5 x 6.5 μm<sup>2</sup>) coupled to a 50μm thick gadolinium scintillator (RC Tritec, 2014), followed  
250 by optimal light magnification using a 35 mm objective (Canon EF 35 mm f/2.0 IS USM).  
251 Furthermore, the camera was cooled down to -30°C, to reduce noise. Pieces of few  
252 centimeters of the extruded samples (T0) were placed in an aluminum film inside a  
253 cylindrical aluminum cell placed in front of the scintillator on the rotating table.

254

### 255 **2.8.2 Image pre-processing and 3D reconstruction**

256 An in house-developed macro from the IMAGINE imaging station and implemented in  
257 ImageJ (Rueden, et al., 2017) was used to normalize the projection images, remove noise  
258 and correct the tilt-angle. Hereinafter, the SYRMEP Tomo Project (STP) (Brun, et al., 2017)  
259 was used to reconstruct a 3D-map of the linear attenuation coefficient (LAC) [cm<sup>-1</sup>] of the  
260 neutrons for the scanned samples. The LAC was reconstructed on 32-bit grey-level scale.

261

### 262 **2.8.3 Image post processing**

263 The reconstructed slices contained two well-separated peaks, associated to air and  
264 starch/*Spirulina*. Standard grey-level thresholding was shown to be unable to accurately  
265 segment the foams, due to the large variance in grey-scale values between the thinner and  
266 thicker cell wall structures, as shown in Figure S1A. Therefore, an automated (supervised)  
267 pixel-level classification method (based on intensity, edge and texture with  $\sigma = 0.7$ )  
268 implemented in the Ilastik software framework (Sommer, Straehle, Koethe, & Hamprecht,  
269 2011) was applied. One slice from each type of sample group was used for combined  
270 training, wherein after the automated segmentation method was applied to all respective  
271 slices of the given data sets, as shown in Figure S1B.

272

273 In extrusion, both the material composition and the utilized processing parameters determine  
274 the pore size and cell wall thickness. Consequently, samples with a very thin outer cell wall  
275 thickness tend to rupture easily, as shown in (Bárbara Franco Lucas, et al., 2018). The open  
276 path between the air outside and inside the sample respectively hampers more advanced  
277 image analysis, especially so called skeletonization, followed by the calculation of the  
278 Connectivity Density (Conn.D). Therefore, an image processing protocol implemented in  
279 ImageJ, which successfully closes the open path at broken cell walls, was developed and  
280 applied to all of the hereby addressed data sets, as shown in Figure S1.

281

#### 282 **2.8.4 Quantitative 3D image analysis**

283 Quantitative 3D image analysis was performed using the Pore3D software library (Brun, et  
284 al., 2010) on Representative Volume of Interest (RVI) of the respective data sets, following  
285 an RVI-test, as further described in the Supplementary Material section ‘Representative  
286 Volume of Interest (RVI) test’. The following image parameters were quantified: 1) Volume  
287 percentage (Vol. Perc.) of the air part, also referred to as ‘porosity’, 2) Specific Surface Area,

288 computed as the quotient between the surface area of the object divided with the total volume  
289 of the VOI as further described by (Parfitt, et al., 1987), 3) Structure Thickness (St.Th.) of  
290 the extruded foams, hereinafter referred to as ‘cell wall thickness’ and 4) Conn.D, as well as  
291 5) pore and 6) throat sizes, based on skeletonization, as further described in (Furlan, et al.,  
292 2018), hereinafter referred to as Method 1. For objective comparison, the pore size was also  
293 estimated on the individually separated macro pores, by applying a ‘Distance Transform  
294 Watershed 3D’ algorithm in the MorphLibJ package (Legland, Arganda-Carreras, &  
295 Andrey, 2016) in ImageJ, using the following parameters: Borgfors(3,4,5) and  
296 ‘normalization dynamic’: 6.0, followed by quantification using Pore3D, hereinafter referred  
297 to as Method 2. However, only Method 1 is capable of estimating both the pore and the  
298 throat size.

299

### 300 **2.8.5 3D Visualization**

301 The 3D volume renderings were performed using the Drishti package for visualization of  
302 volumetric datasets (Limaye, 2012) and using ‘Volume viewer’ in ImageJ (Rueden, et al.,  
303 2017) for visualization of the ‘local thickness’ (Hildebrand & Rüeggsegger, 1997), as well as  
304 the individually separated macro pores, as separated by ‘Wathershedding’.

305

### 306 **2.9 Uniaxial Compression testing**

307 Uniaxial compression tests were performed on an Instron material testing device (model  
308 5542, Instron, Norwood, MA, USA). Extruded samples were carefully cut to cylindrical  
309 shapes with a height of ca. 1 cm with the help of a sharp scalpel and placed between flat  
310 metal surfaces covered with emery paper to avoid slippage. A 3 cm cylindrical probe was  
311 used to compress the samples until fracture, using a 500 N load cell (load values during  
312 compression were in the range of 125-200 N) at a crosshead speed of 1 mm/s. At least 5

313 replicates of each type of extruded sample were measured. Force (N) and distance (mm)  
314 were converted to true stress ( $\sigma_T$ ) and true strain ( $\varepsilon_T$ ) using equations 1 and 2 by the  
315 instrument software Blue Hill.

$$316 \quad \sigma_T = \frac{F(t)(h_0 - \Delta h(t))}{\pi r_0^2 h_0} \quad (4)$$

$$317 \quad \varepsilon_T = \ln\left(\frac{h_0}{h_0 - \Delta h(t)}\right) \quad (5)$$

318 Where  $h_0$  is the initial height of the sample,  $\Delta h(t)$  is the change in height during  
319 compression and  $r_0$  is the initial radius of the sample. The compression modulus was  
320 obtained as the slope of the initial linear zone of the true stress vs. true strain plots. Mean  
321 values of the critical stress and the compression modulus were compared by Tukey and  
322 Fisher tests with a confidence interval of 95% using Excel Stats software.

323

### 324 **3. Results and Discussion**

#### 325 **3.1 Nanostructure of the as-processed foams**

326 In this work, different contents of *Spirulina* (1, 5 and 10 wt.%) were incorporated into corn  
327 starch formulations to produce foams by means of extrusion cooking. To investigate the  
328 effects of the added microalgae on the structure of the foams, the samples were characterized  
329 by wide angle and small angle X-ray scattering (WAXS and SAXS). These techniques are  
330 very powerful tools to extract information on the lamellar and crystalline arrangement of  
331 starch, as widely reported in the literature (Blazek, et al., 2011; P. Jenkins, et al., 1994).

332

333 The WAXS patterns of the raw materials and the as-processed foams (T0) are shown in  
334 Figure 1A. As observed the raw corn starch presented WAXS patterns typical from the A-  
335 type crystalline allomorph, characterized by the main diffraction peaks located at  $2\theta = 15.1^\circ$ ,  
336  $17.0^\circ$ ,  $18.1^\circ$  and  $23.1^\circ$ . On the other hand, the raw *Spirulina* presented several sharp peaks,

337 of which the most intense ones were located at  $2\theta$  values of ca.  $20.6^\circ$ ,  $29.1^\circ$ ,  $29.6^\circ$ ,  $30.9^\circ$ ,  
338  $31.7^\circ$ ,  $38.0^\circ$ ,  $41.8^\circ$  and  $47.1^\circ$  the most intense ones. The appearance of these peaks has been  
339 previously associated to the presence of crystallized fatty acids in this microalga (Martínez-  
340 Sanz, et al., 2018), although they have not been assigned to any particular crystalline  
341 allomorph yet. In fact, the crystallinity of the raw *Spirulina* is very close to the total amount  
342 of lipids determined in the sample (ca. 15%), thus supporting the hypothesis of crystallized  
343 fatty acids present in the native microalga. After the extrusion cooking process, a very  
344 amorphous structure, showing a WAXS pattern resembling the characteristic pattern of the  
345 V-type crystalline allomorph, was observed for the pure starch foam. Interestingly, the  
346 position and relative intensity of the scattering peaks did not correspond to any of the  
347 reported pure V-type crystalline structures. This is in agreement with a previous study which  
348 demonstrated that corn starch samples shortly after being processed by extrusion presented  
349 a non-stable structure consisting of a mixture of different crystalline allomorphs (Benito-  
350 González, López-Rubio, & Martínez-Sanz, 2019). The samples containing *Spirulina* showed  
351 scattering patterns similar to those reported for V-type starches and displayed greater  
352 crystallinity values than the pure starch foam (cf. Table 1). The application of heat/moisture  
353 treatments is known to induce the formation of V-type crystalline structures in corn starch  
354 due to the complexation of the fatty acids and phospholipids naturally present in the raw  
355 cereal with the amylose chains from starch (Kugimiya, Donovan, & Wong, 1980; Zobel,  
356 1988). Our results indicate that the formation of V-type crystallinity was clearly favoured by  
357 the presence of *Spirulina*. The weak cell walls from *Spirulina* have been seen to break down  
358 very easily, even with only mild stirring (Fabra, et al., 2017; Martínez-Sanz, et al., 2018),  
359 thereby enabling the release of the components contained within the cells, such as lipids and  
360 proteins. Furthermore, a previous study showed that the formation of V-type crystallites as  
361 the result of lipid-amylose complexation takes place when subjecting corn starch to a high-



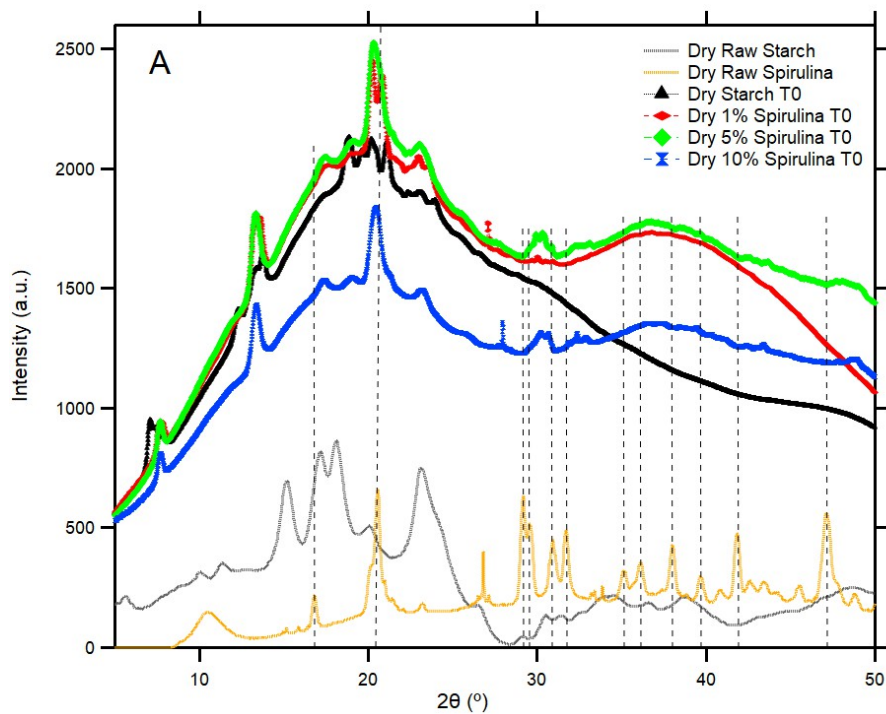
362 humidity thermal treatment in the presence of *Spirulina* (Martínez-Sanz, et al., 2018). Thus,  
363 it can be assumed that the shearing effect during the extrusion cooking process promoted  
364 cell wall disruption and facilitated the release of intracellular components which were able  
365 to form complexes with amylose. Moreover, the WAXS results suggest that the formed V-  
366 type complexes were more thermally stable than the original A-type crystallites present in  
367 the raw corn starch, hence producing slightly more crystalline samples after the extrusion  
368 cooking process. This is in agreement with previous work which reported that a small  
369 fraction of these V-type crystallites was resistant at 110 °C (Martínez-Sanz, et al., 2018).  
370 Accordingly, the WAXS results suggest that, in parallel with the gelatinization process, the  
371 presence of *Spirulina* cell wall components favoured the formation of V-type complexes,  
372 which were more thermally stable than the original A-type crystallites present in the raw  
373 corn starch. This resulted in the production of slightly more crystalline samples after the  
374 extrusion cooking process. It should be noted that none of the peaks detected in the hybrid  
375 foams corresponded to those assigned to the raw microalgae, suggesting that the fatty acid  
376 crystals were melted upon processing.

377  
378 While WAXS is able to provide information on the crystalline structure of starch, SAXS is  
379 mostly used to understand the lamellar arrangement. The SAXS patterns from the raw  
380 materials and the obtained foams (T0) are shown in Figure 1B. As observed, only the native  
381 starch presented a weak and broad lamellar peak centred at ca.  $0.071 \text{ \AA}^{-1}$  in the native dry  
382 state, which corresponds to a lamellar repeat distance of ca. 8.8 nm. The identification of the  
383 starch lamellar peaks is typically much more evident in hydrated samples. This may be due  
384 to the plasticization of amylopectin branch points, increasing the mobility of the amylopectin  
385 double helices, which are then able to align in layers (i.e. lamellae), or simply to increased  
386 contrast between the amorphous and the semicrystalline lamellae as a result of the easier

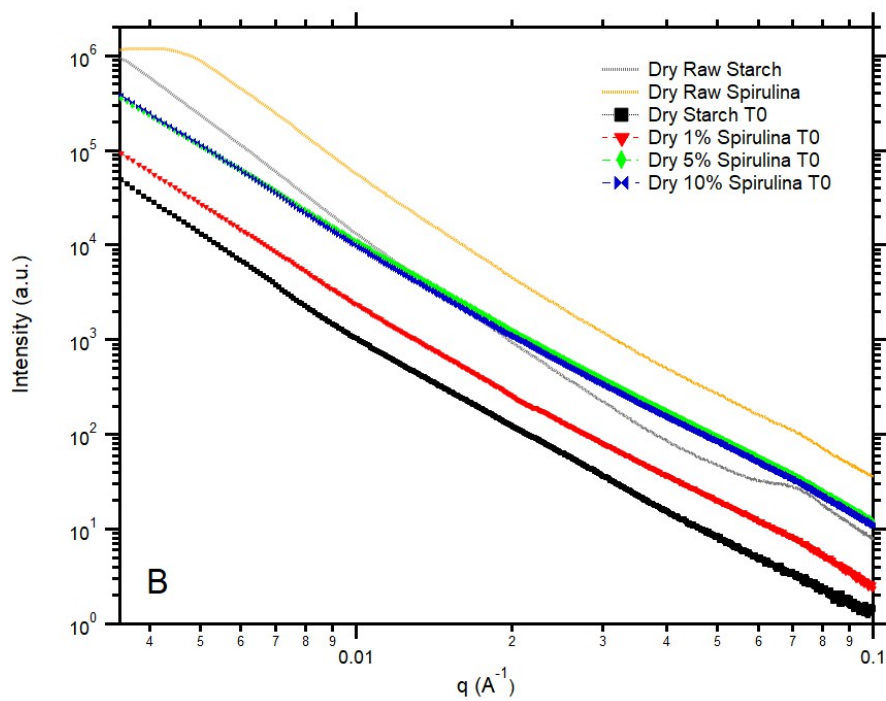
387 access of water towards the amorphous part of the granules. On the contrary, in the dry state  
388 the amylopectin branch points become less flexible and as a result, their arrangement into  
389 lamellae is more limited, although the correlations within the double helices persist (giving  
390 rise to the WAXS peaks). Accordingly, the lamellar peak detected in the raw starch became  
391 more intense and sharp in the hydrated state and its position shifted towards ca.  $0.063 \text{ \AA}^{-1}$   
392 (cf. Figure 1C), corresponding to a lamellar repeat distance of ca. 9.9 nm, in agreement with  
393 the lamellar distances previously reported for other corn starches (P. J. Jenkins, Cameron, &  
394 Donald, 1993; Kuang, et al., 2017; Martínez-Sanz, et al., 2018). This peak was absent in the  
395 patterns from the foams, pointing out towards a disruption of the lamellar structure during  
396 the extrusion cooking process, in line with the WAXS results and in agreement with previous  
397 works (Benito-González, Martínez-Sanz, & Lopez-Rubio, 2018). This was further  
398 confirmed by the change in the power-law exponent from the low  $q$  region ( $0.003\text{-}0.005 \text{ \AA}^{-1}$   
399 <sup>1</sup>), which decreased from ca. 4 for the raw starch (corresponding to the Porod's ideal two-  
400 phase system and typically reported for native starches (Chanvrier, et al., 2007; Lopez-  
401 Rubio, Htoon, & Gilbert, 2007)) to 2.3-2.7 for the foams (corresponding to mass fractal  
402 structures). Power-law exponents close to 2 have been reported for gelatinized starches  
403 where the lamellar order was completely lost (Lopez-Rubio, et al., 2007), while slightly  
404 higher exponents, similar to the values here reported, have been estimated for melt-  
405 compounded cellulose-starch samples (Benito-González, et al., 2018). Extrusion of starch  
406 samples in excess water has been reported to completely destroy the lamellar structure  
407 (Waigh, Gidley, Komanshek & Donald, 2000), whereas a weak 9-10 nm lamellar peak has  
408 been seen to remain when the extrusion process was carried out at relatively low water  
409 contents (Chanvrier, Uthayakumaran, Appelqvist, Gidley, Gilbert & López-Rubio, 2007).  
410 Our results support the fact that the lamellar structure of starch was extensively disrupted

411 with the addition of 30 wt.% moisture and the conditions used for the extrusion cooking  
412 process, both for the pure starch and the blends with the added *Spirulina*.

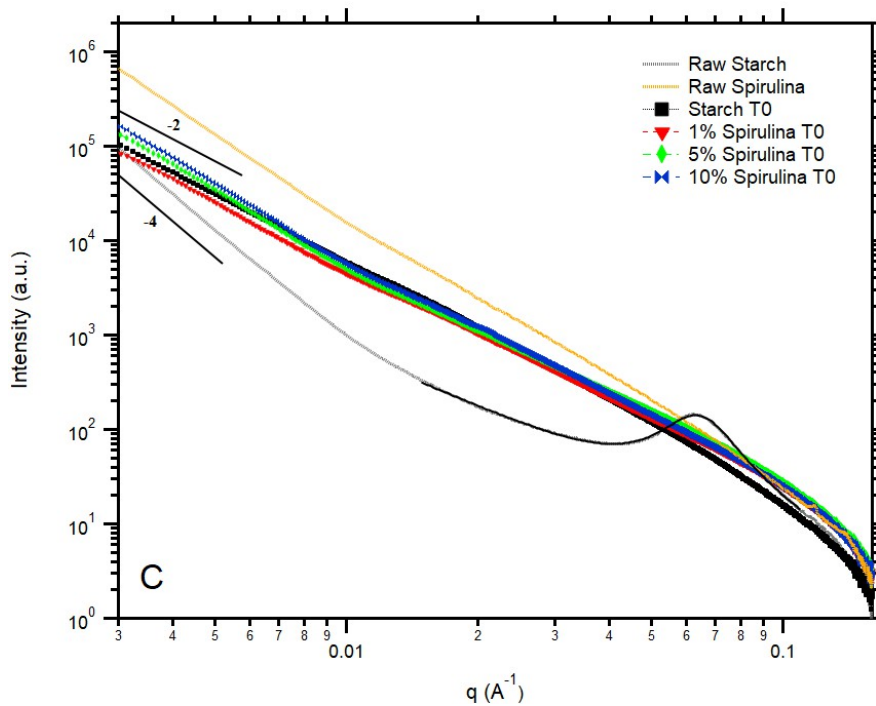
413



414



415



416

417 **Figure 1.** (A) WAXS patterns from the raw starch and *Spirulina* and from the as-processed  
 418 foams (T0). Curves from raw starch and *Spirulina* have been offset for clarity. (B) SAXS  
 419 patterns of the raw starch and *Spirulina* and the foams after being processed (T0) in their  
 420 native dry state and (C) SAXS patterns from the fully hydrated samples. Dotted lines  
 421 correspond to the experimental data, whereas solid lines correspond to the fitting curves.

422

423 **Table 1.** Power-law exponents, lamellar peak parameters and crystallinity ( $X_C$ ) for the raw  
 424 materials and the foams after being processed (T0). The lamellar peak parameters were  
 425 obtained by fitting the experimental SAXS data of the hydrated samples to the sum function  
 426 of a power-law plus a Gaussian-Lorentzian peak and crystallinity was obtained from the  
 427 peak fitting of the WAXS patterns from the dry samples. Standard deviation values on the  
 428 last digit are shown in parentheses for the fitting parameters.

Power-law exponent	Lamellar peak position ( $\text{\AA}^{-1}$ )	Width of the lamellar peak ( $\text{\AA}^{-1}$ )	Lamellar repeat distance ( $\text{\AA}$ )	$X_C$ (%)
--------------------	--	--	---	-----------

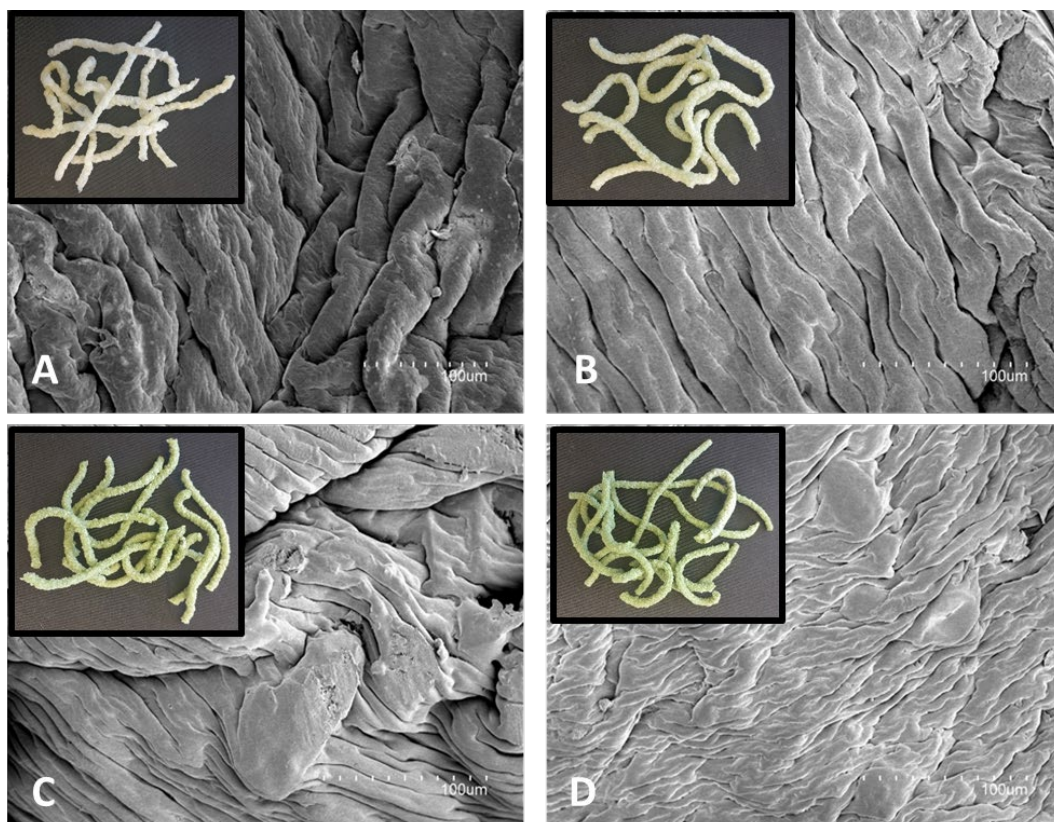
Raw <i>Spirulina</i>	3.24 (1)	---	---	---	14.98
Raw Starch	2.009 (3)	0.06335 (1)	0.02543 (5)	9.92	42.71
Starch	2.49 (1)	---	---	---	3.22
1% <i>Spirulina</i>	2.34 (5)	---	---	---	4.59
5% <i>Spirulina</i>	2.52 (1)	---	---	---	6.62
10% <i>Spirulina</i>	2.73 (1)	---	---	---	8.72

429

430 **3.2 Microstructure and mechanical properties of the as-processed foams**

431

432 To understand the effects of *Spirulina* addition at a larger size range, the microstructure of  
433 the obtained foams was also investigated by SEM and neutron tomography. Representative  
434 SEM images, together with pictures showing the visual appearance of the foams, are shown  
435 in Figure 2. As observed, the addition of *Spirulina* produced foams with a greenish  
436 coloration, which was more evident for loadings higher than 5 wt.%. Furthermore, as  
437 evidenced by the SEM images, more densely packed structures were present in the foams  
438 containing higher *Spirulina* loadings. Such effect that was also observed macroscopically.  
439

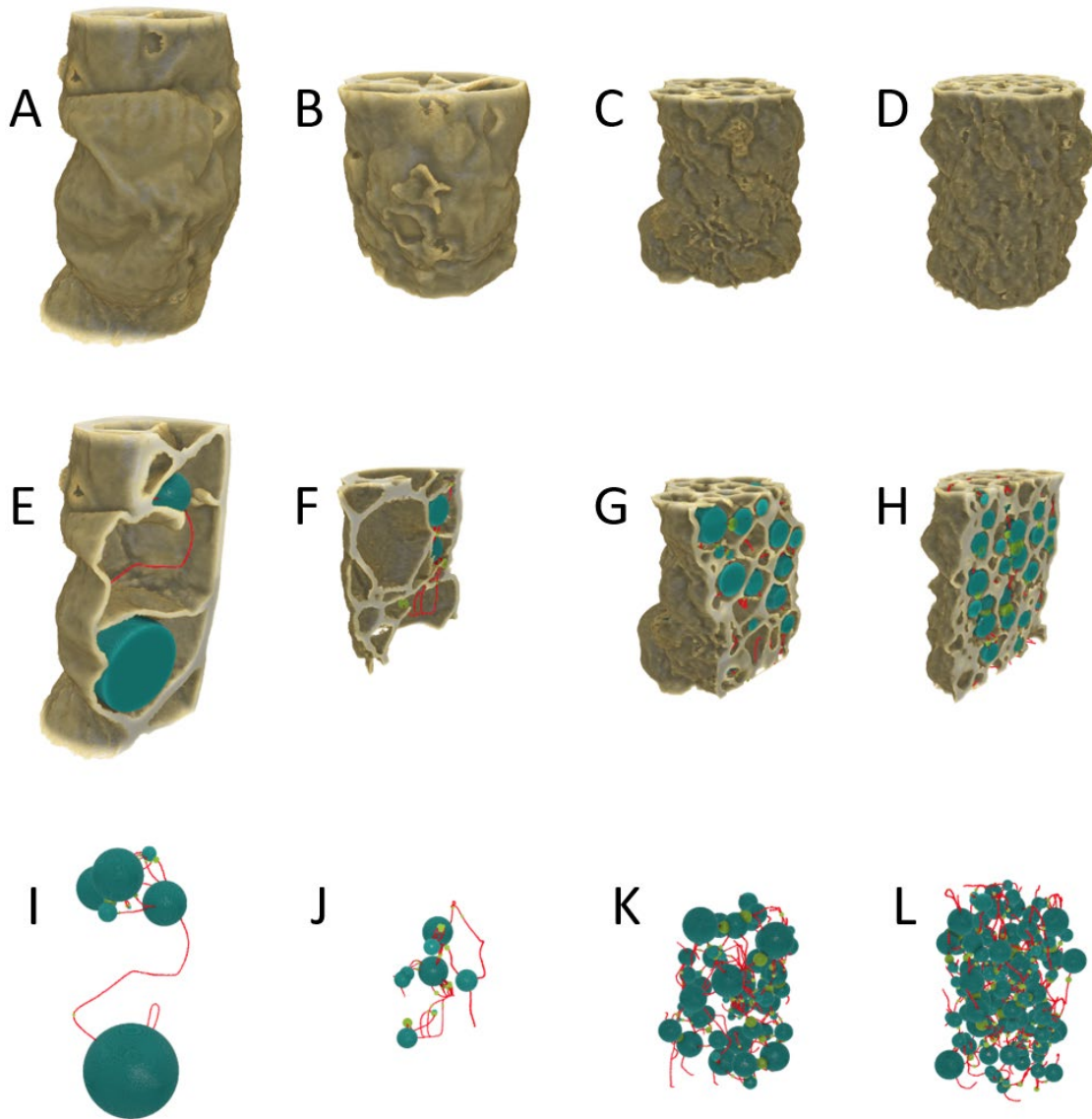


440  
441 **Figure 2.** SEM micrographs from the as-processed foams (T0): (A) Pure starch, (B) 1%  
442 *Spirulina*, (C) 5% *Spirulina* and (D) 10% *Spirulina*. Image inserts show the visual  
443 appearance of the foams.

444

445 An in-depth 3D characterization of the microstructure from the different foams was carried  
446 out by means of neutron tomography. The porous structure of the extruded foams could be  
447 clearly resolved and visualized by this technique, as shown in Figure 3 and Figure 4. The  
448 visual results were also supported with quantitative image analysis and the obtained  
449 parameters are summarized in Table 2. As observed, loadings higher than 5 wt.% *Spirulina*  
450 led to a reduction in the porosity. The specific surface area and connectivity density  
451 (Conn.D) increased with increasing concentrations of *Spirulina*, indicating that more well-  
452 connected foams were formed. In addition, the cell wall thickness decreased with the  
453 addition of *Spirulina*, although there was no clear trend with the microalga concentration  
454 (cf. Table 2 and Figure 4). However, no significant differences in pore size or throat size  
455 could be measured, due to the large heterogeneity of the samples, which led to large standard  
456 deviations. These data have thus been supported by pore and throat size probability  
457 distributions, calculated with method 1 and 2, as described in the Supplementary Material  
458 section (Figures S4, S5 and S6), indicating that the pore size (mean and standard deviation)  
459 decreased with increasing concentration of *Spirulina*.

460



461

462 **Figure 3.** Visualization of the microstructure of the as-processed foams (T0) determined by  
 463 neutron tomography. (A-D) show volume renderings of the extruded foams (in light brown),  
 464 (E-H) with applied virtual cut planes. (E-H and I-L) show the longest connected image  
 465 skeleton (in red) throughout the pore space, with maximal inscribed blobs in the porous part  
 466 at the nodes (dark green) and at the throats (light green) along the longest skeleton, as  
 467 described by ‘Method 1’. The height of the samples were 11.2 mm and 7.1 mm for the pure  
 468 starch and the hybrid starch-*Spirulina* samples, respectively. (A, E, I) Pure starch, (B, F, J)  
 469 1% *Spirulina*, (C, G, K) 5% *Spirulina* and (D, H, L) 10% *Spirulina*.

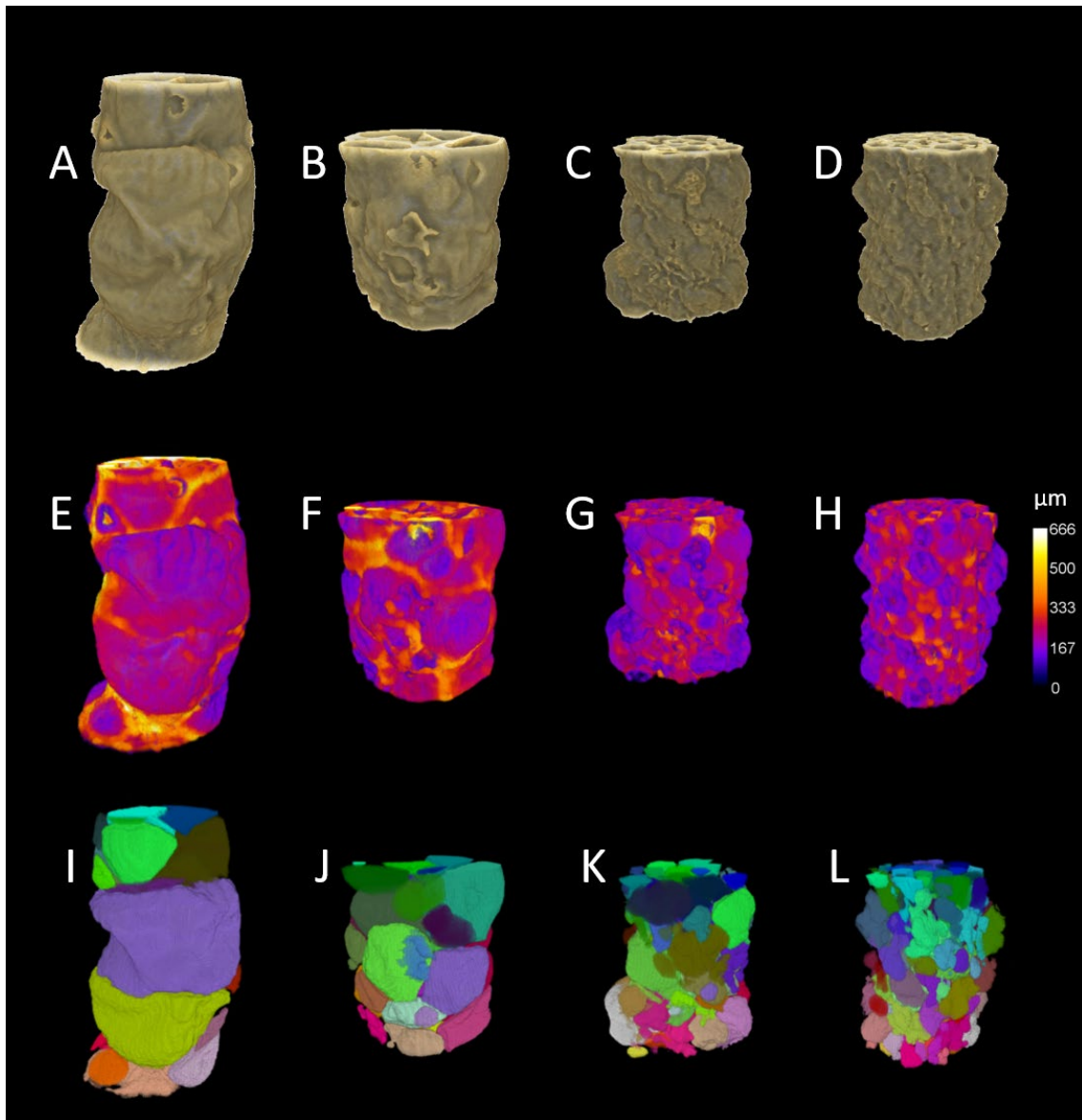


470

471 **Table 2.** Extracted image descriptors obtained by applying quantitative 3D image analysis  
 472 on selected volumes of interests (VOIs), with a representative VOI height of 11.2 mm and  
 473 7.1 mm for the pure starch and the hybrid starch-*Spirulina* samples, respectively. See Figures  
 474 S2 and S3 for details of how the representative volume of interest was selected.

	Porosity (%)	Specific Surface Area (mm <sup>-1</sup> )	Cell wall thickness (μm)	Conn.D (mm <sup>-3</sup> )	Pore Size (μm) Method 1 (Method 2)	Throat Size (μm) Method 1
Starch	67.6 ± 3.1	1.7 ± 0.1	234.0 ± 18.4	0.06 ± 0.00	1109.0 ± 1068.9 (1210.9 ± 922.1)	161.2 ± 114.9
1% <i>Spirulina</i>	64.8 ± 1.2	2.5 ± 0.1	199.1 ± 0.2	0.07 ± 0.00	707.7 ± 527.4 (859.9 ± 600.6)	178.0 ± 133.2
5% <i>Spirulina</i>	57.2 ± 2.4	3.8 ± 0.1	165.1 ± 1.7	0.34 ± 0.05	652.8 ± 279.1 (671.3 ± 247.9)	152.9 ± 91.9
10% <i>Spirulina</i>	50.3 ± 5.0	4.2 ± 0.2	180.1 ± 25.2	0.79 ± 0.09	528.5 ± 198.4 (574.4 ± 196.3)	159.0 ± 92.5

475



476

477 **Figure 4.** (A-D) Volume renderings of the as-processed foams (T0) (in light brown), as  
 478 determined by neutron tomography. (E-H) Visualization of the cell wall thickness, which is  
 479 colour coded, and given in  $\mu\text{m}$ . (I-L) Visualization of the individual pores, following 3D  
 480 water shedding and colour labelling, as described by ‘Method 2’. The height of the samples  
 481 were 11.2 mm and 7.1 mm for the starch and hybrid starch-*Spirulina* foams, respectively.  
 482 (A, E, I) Pure starch, (B, F, J) 1% *Spirulina*, (C, G, K) 5% *Spirulina* and (D, H, L) 10%  
 483 *Spirulina*.

484

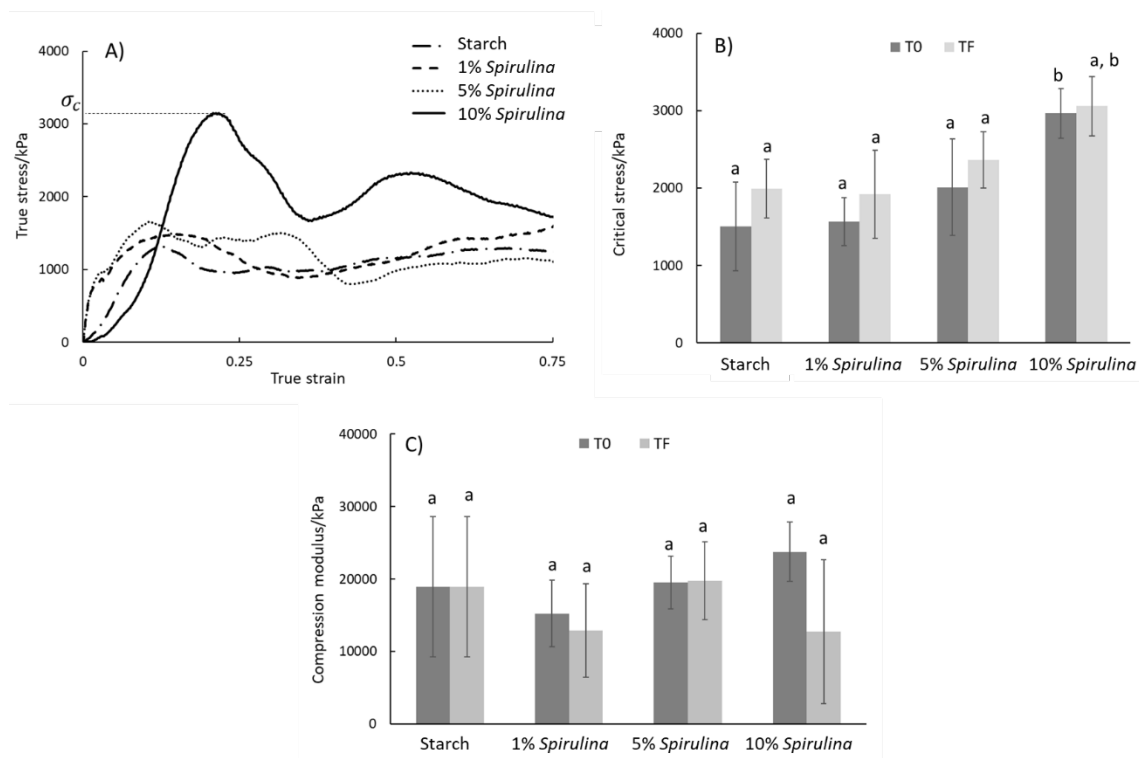
485 The structural changes undergone by starch during the extrusion cooking process were  
486 expected to have a strong impact on the mechanical properties of the obtained foams, which  
487 in turn are directly linked to their texture. Food texture is a very important attribute from the  
488 perspective of consumers' acceptance and thus, it is crucial for the application of these foams  
489 as food snacks. The expansion index of the extrudates is directly related to the microstructure  
490 of extrudates, and has been shown to be a determining factor for the crunchiness (Joshi, et  
491 al., 2014). The expansion index was calculated as described in the materials and methods  
492 section and was reduced from  $3.1 \pm 0.2$  for the corn starch to  $2.8 \pm 0.1$ ,  $2.3 \pm 0.1$  and  $2.2 \pm$   
493  $0.1$  for the hybrid samples containing 1%, 5% and 10 % *Spirulina*, respectively. These results  
494 are in agreement with previous works which showed no effect on the expansion index of  
495 rice and corn flour mixtures (2:1) with the addition of 0.4-2.6 wt.% *Spirulina* (reporting  
496 values which ranged from 2.5 to 4.5 depending on the extrusion conditions) (Bárbara  
497 Franco Lucas, et al., 2018; Barbara Franco Lucas, et al., 2017; Morsy, et al., 2014), while  
498 higher *Spirulina* loadings (6-8%) have been shown to reduce the expansion index (Tańska,  
499 Konopka, & Ruszkowska, 2017). As suggested by the WAXS results, the *Spirulina* cell wall  
500 components were able to form complexes with amylose during the extrusion cooking  
501 process, leading to the formation of more thermally-stable V-type crystallites and, therefore,  
502 producing slightly more crystalline foams with reduced elasticity and thus, limited ability to  
503 expand. The reduced expansion index of the 5% and 10% *Spirulina* hybrid foams can be  
504 directly linked to their more densely packed structures, as observed by SEM and neutron  
505 tomography.

506

507 Compression experiments were also carried out to determine the mechanical properties of  
508 the as-processed (T0) foams and typical compression true stress-strain curves are shown in  
509 Figure 5A. A critical value of stress ( $\sigma_c$ ), defined as the first maximum stress value, could

510 be identified for all the samples, but more clearly for the 10% *Spirulina* foam. After the  
511 critical stress it was observed that the samples followed a brittle fracture as the compression  
512 test progressed, indicated by the curve pattern with peaks up and down until the sample  
513 collapses characteristic of this type of systems (Attenburrow, Goodband, Taylor, & Lillford,  
514 1989). As deduced from Figure 5B, only the highest *Spirulina* loading resulted in a  
515 significant increase (95% confidence interval) in the critical stress ( $2966 \pm 322$  kPa for 10%  
516 *Spirulina*, as compared to  $1505 \pm 573$  kPa,  $1568 \pm 310$  kPa and  $2011 \pm 622$  kPa for the pure  
517 starch, 1% *Spirulina* and 5% *Spirulina*, respectively). A similar trend of increased hardness  
518 has been previously reported with the addition of lower *Spirulina* contents into extruded  
519 starch (Barbara Franco Lucas, et al., 2017) and was attributed to the effect of proteins in  
520 restricting moisture accessibility towards starch. The slightly greater crystallinity of the  
521 samples containing *Spirulina* can also explain the hardness increase. On the other hand, the  
522 stiffness of the foams was not significantly affected, as suggested by their compression  
523 moduli (15000-25000 kPa) (cf. Figure 5C), which were within the range of previously  
524 reported values for extruded starch foams (Robin, et al., 2010). It should be noted that the  
525 larger standard deviation values for the pure starch foam reflect a greater structural  
526 heterogeneity. As previously discussed, the microstructure of the extruded samples consisted  
527 of a porous network which may be described as a solid foam (cf. Figure 2 and Figure 3). The  
528 micromechanics of foam deformation during compression starts by cell walls bending  
529 elastically, and giving rise to the initial linear region in the stress-strain curves, followed by  
530 structural collapse when a critical value of stress is reached (Ashby & Medalist, 1983). Ashby  
531 and Gibson demonstrated that the ratio of the bulk density to the density of the cell wall  
532 material can be correlated to the critical stress and modulus of foams (Ashby, et al., 1983).  
533 The strongest sample (i.e. presenting the highest critical stress) would correspond to that  
534 with the greater bulk density and containing thinner cell walls and a higher number of cells

535 (10% *Spirulina*), whilst coarse structures with a lower number of cells would be the weakest  
 536 (pure starch and 1% *Spirulina*). The increased hardness of the 10% *Spirulina* foam is  
 537 expected to have some implications on the consumers' acceptance. This will be further  
 538 evaluated by sensory analyses in the future.  
 539



540  
 541 **Figure 5.** (A) Representative true stress vs strain curves of starch-*Spirulina* foams  
 542 conditioned at 30% RH. (B) Average critical stress for samples before (T0) and after storage  
 543 (TF). (C) Average compression modulus of the extruded samples before (T0) and after  
 544 storage (TF). Different letters indicate statistically significant differences between samples  
 545 ( $p \leq 0.05$ ).

546  
 547 **3.2 Effect of retrogradation upon prolonged storage**

548 After being gelatinized, starch is capable of re-crystallizing upon storage at certain  
 549 conditions. During this re-crystallization process, known as retrogradation, the amylose

550 chains gradually re-associate and re-organize to form a different ordered structure (Wang,  
551 Li, Copeland, Niu, & Wang, 2015). The impact of the *Spirulina* microalga on the  
552 retrogradation process was evaluated by characterizing the obtained foams after storage at  
553 30% RH for one month (TF). This would be representative of the properties of the foams  
554 during their shelf-life. The nanostructural changes undergone by the samples at TF were  
555 investigated by means of WAXS and SAXS. The WAXS patterns from the stored foams are  
556 shown in Figure 6A. As observed, the crystalline structure of the foams changed from the  
557 non-stable V-type mixture observed shortly after processing the samples (T0) towards a V<sub>A</sub>  
558 type structure, in agreement with what has been previously reported for other extruded starch  
559 samples (Benito-González, et al., 2019; J. J. Van Soest, Hulleman, De Wit, & Vliegenthart,  
560 1996). Additionally, two peaks located at ca. 17° and 22°, which are characteristic from the  
561 B-type allomorph, were detected, suggesting that a certain fraction of starch re-crystallized  
562 into this type of structure. Corn starch has been reported to re-crystallize into a B-type  
563 structure when stored at low temperature (Shamai et al. 2003) or high relative humidity  
564 conditions (Fu et al. 2013) and similar results have also been reported for corn starch samples  
565 processed by means of melt compounding in the presence of 50 wt.% moisture (Benito-  
566 González, et al., 2019). The re-crystallization into B-type structures has been related to the  
567 rearrangement and association of the outermost short branches of amylopectin (Hoover,  
568 Hughes, Chung, & Liu, 2010). The crystallinity values calculated for the foams at TF (cf.  
569 Table 3) were very far from the value corresponding to the native starch (cf. Table 1) and  
570 indicate that only a small fraction of starch was able to re-crystallize upon storage at  
571 30% RH. The pure extruded starch and the hybrid foams presented very similar  
572 crystallinities at TF and only the highest *Spirulina* loading gave rise to a slight crystallinity  
573 increase compared to the pure starch. Comparing with the values estimated just after  
574 processing (T0), the retrogradation process gave rise to a crystallinity increase of 92% in the

575 case of pure starch and 23% for the 1% *Spirulina* foam, whereas the crystallinity  
576 corresponding to the starch fraction was slightly decreased for the 5% and 10% *Spirulina*  
577 foams. It is known that greater moisture contents favour starch re-crystallization (J. Van  
578 Soest & Knooren, 1997; Wang, et al., 2015) and in particular, the presence of moisture is  
579 necessary for the transition of V-type complexes into B-type crystallites ((Kugimiya, et al.,  
580 1980; Zobel, 1988). Previous studies have pointed out that the proteins present in *Spirulina*  
581 may compete for water binding sites, hindering the hydration of starch granules (Batista, et  
582 al., 2012; Barbara Franco Lucas, et al., 2017; Tańska, et al., 2017). These results confirm the  
583 fact that the presence of the microalga limited the accessibility of moisture towards the starch  
584 molecules and as a result, hindered the re-crystallization process. A similar effect has been  
585 attributed to the presence of cellulose (Benito-González, et al., 2019) and other fillers which  
586 interact with the hydroxyl groups from starch by the formation of hydrogen bonds (Ferrero,  
587 Martino, & Zaritzky, 1994; Ghanbarzadeh, Almasi, & Entezami, 2011). It should be noted  
588 that the 5% *Spirulina* and 10% *Spirulina* foams displayed additional weak peaks which  
589 corresponded to the most intense scattering peaks from the raw *Spirulina*. This indicates that  
590 the free fatty acids contained in the microalga were able to re-crystallize back to their native  
591 form upon storage of the foams.

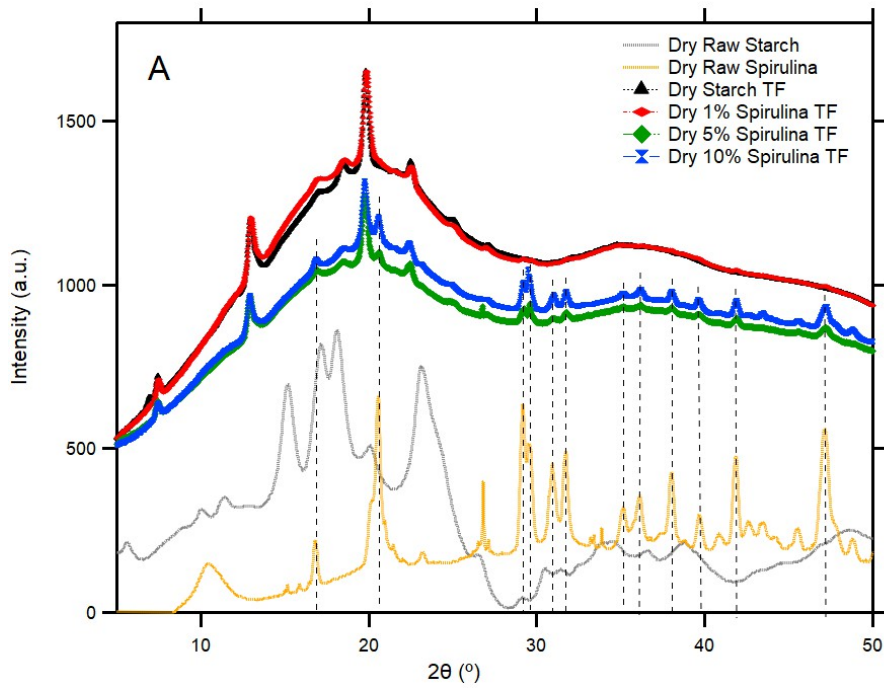
592  
593 To confirm the effect of *Spirulina* on the crystallinity of the foams after storage, FT-IR  
594 analyses were also carried out and the obtained spectra are shown in Figure S7. The spectral  
595 region of 800–1200  $\text{cm}^{-1}$  was used to investigate structural differences between samples, as  
596 previous studies have suggested that bands in this ‘fingerprint’ region reflect changes in  
597 polymer conformation and hydration of processed starches (Capron, Robert, Colonna,  
598 Brogly, & Planchot, 2007; Htoon, et al., 2009; Sevenou, Hill, Farhat, & Mitchell, 2002; J. J.  
599 G. van Soest & Vliegenthart, 1997). The band at 1022  $\text{cm}^{-1}$  has been seen to increase in

600 more amorphous samples, while the bands at 1000 and 1047  $\text{cm}^{-1}$  become more defined in  
601 more crystalline samples (Capron, et al., 2007; Sevenou, et al., 2002; J. J. G. van Soest, et  
602 al., 1997). In particular, the trend in the intensity ratio for the 1022:1000 bands has been  
603 reported to correlate reasonably with the crystallinity measured by X-ray diffraction  
604 (Capron, et al., 2007). The values here obtained for the 1022:1000 intensity ratio were  
605 approximately constant (ca. 0.96-0.94) for all the samples, confirming that starch presented  
606 a similar degree of crystallinity in all the foams after storage for one month.

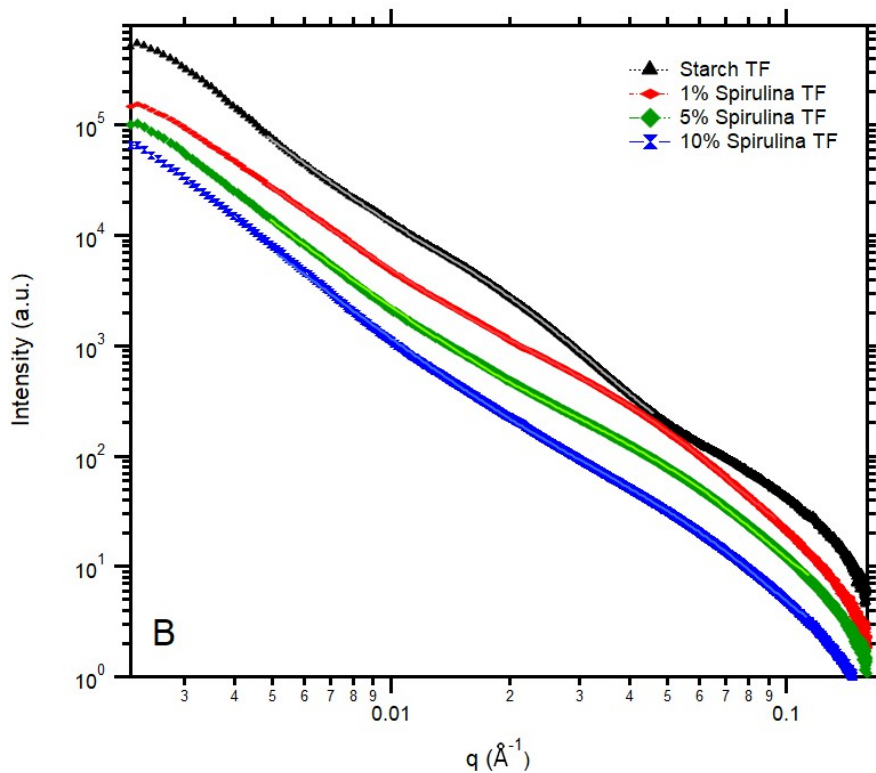
607  
608 Very broad and weak shoulders were detected in the SAXS patterns from the foams after  
609 storage (cf. Figure 6B). This type of scattering feature has been previously detected for  
610 retrograded starches and has been assigned to the formation of heterogeneous semi-crystalline  
611 structures (Shamai, Shimoni, & Bianco-Peled, 2004; Suzuki, Chiba, & Yarno, 1997). The  
612 associated lamellar repeat distances ranged from ca. 51 nm for the pure starch to ca. 40-44  
613 nm for the hybrid foams. Suzuki et al. reported lamellar distances of ca. 15-30 nm for potato  
614 starch retrograded at low temperature (Suzuki, et al., 1997), whereas Shamai et al. noted the  
615 appearance of a broad lamellar peak, characteristic of distances of ca. 20 nm, in high amylose  
616 corn starch only when the samples were retrograded at low temperatures (Shamai, et al.,  
617 2004). The broader shoulders observed for the hybrid foams indicate that the presence of  
618 *Spirulina* promoted the re-crystallization of starch into more heterogeneous structures.  
619 Furthermore, the power-law exponents in the low  $q$  region were 2.6-2.7 for all the samples,  
620 confirming the existence of structures with low degree of order in the nanometre size range.  
621 The slight increase in the power-law exponents of the foams at TF with respect to the  
622 corresponding values at T0 confirm that the gelatinization process was irreversible and that  
623 the neither the crystalline nor the lamellar structure of native starch were recovered upon  
624 retrogradation. Presence of *Spirulina* and the effect of limited moisture accessibility



625 hindered the re-organization of amylose chains into crystalline domains and lamellae.  
626 However, all the foams presented very similar crystallinity values after storage since the  
627 mentioned effect upon storage was counteracted by the initially greater crystallinity of the  
628 hybrid foams at T0.  
629



630



631

632 **Figure 6.** (A) WAXS patterns from the raw starch and *Spirulina* and from the foams after  
 633 storage for one month (TF) at 30% RH. Curves from raw starch and *Spirulina* have been  
 634 offset for clarity. (B) SAXS patterns from the fully hydrated samples. Dotted lines  
 635 correspond to the experimental data, whereas solid lines correspond to the fitting curves. The  
 636 curves have been offset for clarity.

637

638 **Table 3.** Power-law exponents, lamellar peak parameters and crystallinity ( $X_C$ ) for the foams  
 639 after storage for one month (TF). The lamellar peak parameters were obtained by fitting the  
 640 experimental SAXS data of the hydrated samples to the sum function of a power-law plus a  
 641 Gaussian-Lorentzian peak and crystallinity was obtained from the peak fitting of the WAXS  
 642 patterns from the dry samples. Standard deviation values on the last digit are shown in  
 643 parentheses for the fitting parameters.

	Power-law exponent	Lamellar peak position ( $\text{\AA}^{-1}$ )	Width of the lamellar peak ( $\text{\AA}^{-1}$ )	Lamellar repeat distance ( $\text{\AA}$ )	$X_c$ (%) <sup>(*)</sup>
Starch	2.678 (9)	0.0122 (1)	0.0141 (1)	51.40	6.19 [6.19]
1% <i>Spirulina</i>	2.561 (7)	0.0156 (4)	0.0346 (1)	40.28	5.65 [5.65]
5% <i>Spirulina</i>	2.680 (2)	0.0144 (4)	0.0441 (1)	43.72	6.24 [5.90]
10% <i>Spirulina</i>	2.716 (4)	0.0143 (6)	0.0454 (1)	43.87	8.57 [7.75]

644 <sup>(\*)</sup>The crystallinity values calculated accounting only for the contribution of starch are shown  
645 in brackets.

646

647 As observed in Figure 5B, although the differences were not significant, partially due to a  
648 large variability between replicates, a trend of increased hardness (i.e. greater critical stress  
649 values) was observed when comparing the samples at T0 and TF, which could be expected  
650 from the structural changes occurring during storage (i.e. the amorphous as-processed starch  
651 foam may re-organize into more ordered structures upon storage, leading to the formation of  
652 more mechanically resistant foams). The weak effect is reasonable considering the very  
653 limited re-crystallization of starch after storage. The same trend observed at T0 was detected  
654 for the foams after storage, i.e. the presence of higher amounts of *Spirulina* led to the  
655 formation of harder foams, although the high standard deviation values for the measured  
656 samples preclude from extracting definitive conclusions. Regarding stiffness, no significant  
657 differences were observed between samples before and after storage, partly due to the large  
658 variability between samples.

659

660 Since the crystalline structure of starch is known to have a significant impact on its  
661 digestibility, the content of resistant starch (RS) was determined in the foams at TF,  
662 obtaining values of  $1.1 \pm 0.3$  g RS/100 g sample in the pure starch and the samples with 1%

663 and 5% *Spirulina* and a higher content of  $2.1 \pm 0.7$  g RS/100 g sample in the foam with 10%  
664 *Spirulina*. This effect might be related to the formation of V-type amylose-lipid complexes,  
665 which has been previously noted in starch-*Spirulina* blends (Martínez-Sanz, et al., 2018) and  
666 as suggested by the WAXS patterns of the as-processed foams. These complexes are known  
667 to form more stable crystalline structures than those found in native starches. For instance,  
668 the formation of V-type amylose-lipid complexes has been shown to produce a decrease in  
669 the amylose solubility, hence increasing the gelatinisation temperature (Eliasson, Carlson,  
670 & Larsson, 1981). Furthermore, although amylose-lipid complexes are hydrolysed and  
671 absorbed in the gastrointestinal tract to the same extent as free amylose, the hydrolysis  
672 process has been seen to take place at a slower rate (J. Holm, et al., 1983). From a food  
673 design point of view, a higher content of resistant starch is desirable, since it is related to  
674 lower calorie content and lower glycaemic loads (Fuentes-Zaragoza, Riquelme-Navarrete,  
675 Sánchez-Zapata, & Pérez-Álvarez, 2010). Thus, the addition of 10% *Spirulina* could be  
676 interesting from a nutritional perspective, not only due to the increased protein content and  
677 the antioxidant properties provided by bioactive compounds such as polyphenols (De Marco,  
678 et al., 2014), but also due to the potential benefits associated to an increased content of  
679 resistant starch in the foams.

680

#### 681 **4. Conclusions**

682 Starch foams with different loadings (1, 5 and 10 wt.%) of the microalga *Spirulina* were  
683 produced by means of extrusion cooking. The gelatinization process taking place during the  
684 extrusion process led to the disruption of the crystalline and lamellar structure of native  
685 starch, producing very amorphous foams. Furthermore, the crystallized fatty acids present  
686 in the raw *Spirulina* were completely melted. The release of *Spirulina* intracellular

687 components promoted the formation of more stable V-type complexes with amylose, leaving  
688 slightly more crystalline foams after processing. The microstructure of the foams was  
689 strongly affected by the presence of the microalga and the hybrid foams exhibited more  
690 densely packed and well-connected porous structures. This, in turn, affected the textural  
691 properties of the foams, which became harder with higher *Spirulina* loadings.

692  
693 All the foams presented a very limited re-crystallization upon storage, being this further  
694 reduced in the case of the hybrid foams. As a result, all the foams presented similar  
695 crystallinity values after storage. Furthermore, the starch lamellar arrangement was only  
696 partially recovered, forming much more heterogeneous structures than those found in the  
697 native starch. The formation of amylose complexes was translated into a higher resistant  
698 starch content in the 10% *Spirulina* foam. This, together with the increased protein content  
699 and the presence of bioactive compounds such as polyphenols, highlights the potential  
700 benefits of adding *Spirulina* into starch extruded foams to produce snack foods with higher  
701 nutritional value.

702  
703 These results show that the combination of advanced characterization tools such as X-ray  
704 scattering techniques and neutron tomography can provide very valuable information on the  
705 nano- and microstructure of starch-based products, which will ultimately enable a rational  
706 design of food products with the desired characteristics according to the consumers'  
707 requirements.

708  
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717

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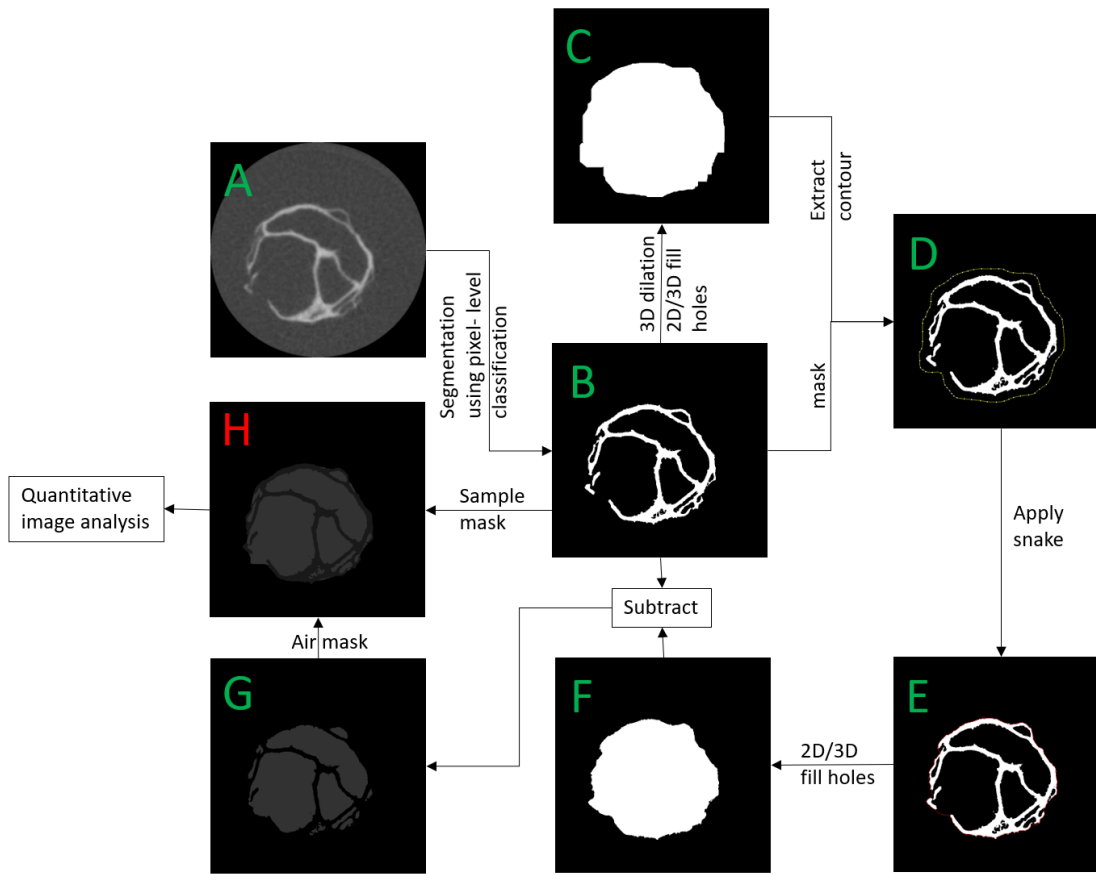


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940

## 941 **Supplementary Material**



942

943 **Figure S1.** Schematic drawing of the applied 2D/3D image processing protocol in order to  
 944 segment the extruded samples with broken cell walls experiencing an open-air macro pore  
 945 connection, thus allowing further quantitative 3D image analysis. The original image (A)  
 946 with a varying grey-scale value for the thinner and thicker cell wall, as well as with  
 947 physically broken cell wall, is segmented using a pixel-level classification method  
 948 implemented in ‘Ilastik’. The resulting image (B) shows a pore connected to the outer air,  
 949 via a thin broken cell wall, which impedes further 3D quantification of the air skeleton and  
 950 quantification of both the connectivity density and pore size distribution. (C-G) shows the  
 951 utilized image processing steps, finally resulting in image (H), where the extruded sample  
 952 and the air pore space have been successfully segmented from the background. All image

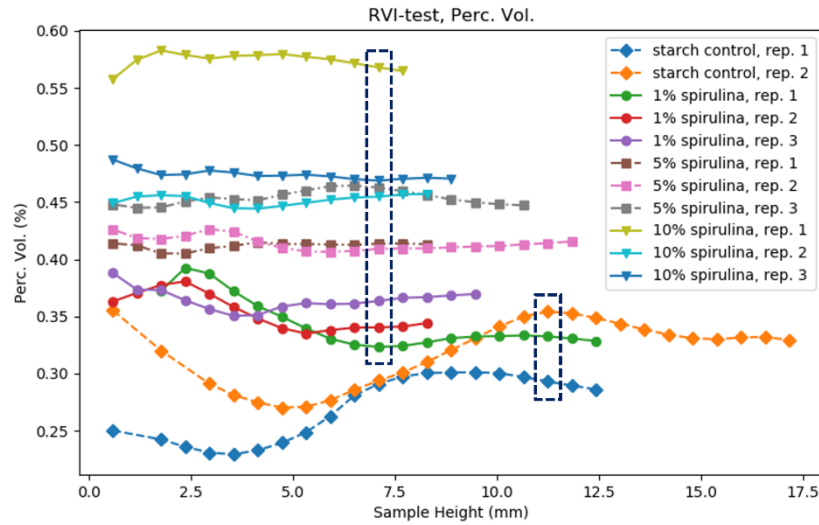
953 processing steps were performed in 3D. The results of these steps were used for both  
954 quantitative image analysis and 3D-renderings.

955

### 956 **Representative Volume of Interest (RVI) test**

957 Quantitative 3D image analysis is commonly applied on so called volume of interests  
958 (VOIs), which are often cubic, and extracted from different positions inside the sample, far  
959 away from sample borders, which can often be non-representative to include in such an  
960 analysis. The size of the considered VOI is determined by a prior Representative Volume of  
961 Interest test (RVI-test) (Bear, 2013), where the quantified parameters, e.g. Volume  
962 percentage (Vol.Perc.) and Connectivity Density (Conn.D) are plotted against the side length  
963 of the VOI, as shown in Figures S2 and S3. The representative VOI-size is found when the  
964 observed parameters ‘level out’ and are no longer affected by the varying size of the  
965 considered VOI (Bear, 2013). Such an approach also works well on extruded samples with  
966 a rather low porosity and a thick cell wall structure of the extrudate, as shown in (Karlsson,  
967 Larsson, Lorén, Stading, & Rigdahl, 2019). However, for extruded samples with a very high  
968 porosity, large pore size and very thin cell wall structure, as for the hereby described  
969 extruded foams with a high starch content, quantitative analysis based on cubic VOIs is  
970 challenging, due to the fact that the quantified parameters fail to ‘level out’, even for the  
971 maximum possible applied VOI-size, following the RVI-test. Therefore, a modified RVI-  
972 test, where the sample diameter is kept ‘as is’ and only the height of the VOI is allowed to  
973 vary, was performed. For the given samples, a representative VOI height of 11.2 mm and  
974 7.1 and was found for the pure starch and hybrid starch-*Spirulina* foams, respectively, as  
975 shown in Figures S2 and S3.

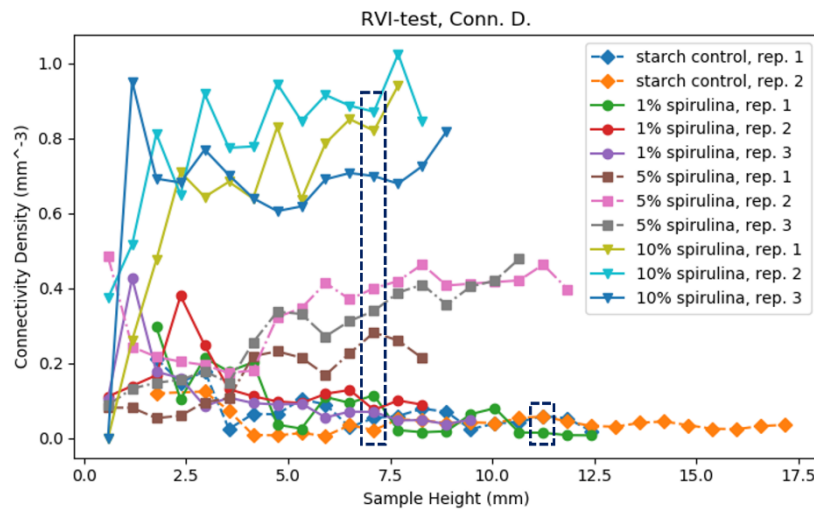
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977

978 **Figure S2.** Test for finding the representative volume of interest (RVI-test), based on the  
 979 Vol. Perc. as the evaluation parameter. The optimal height of the VOI was chosen at 11.2  
 980 mm and 7.1 mm for the starch and hybrid starch-*Spirulina* foams, respectively.

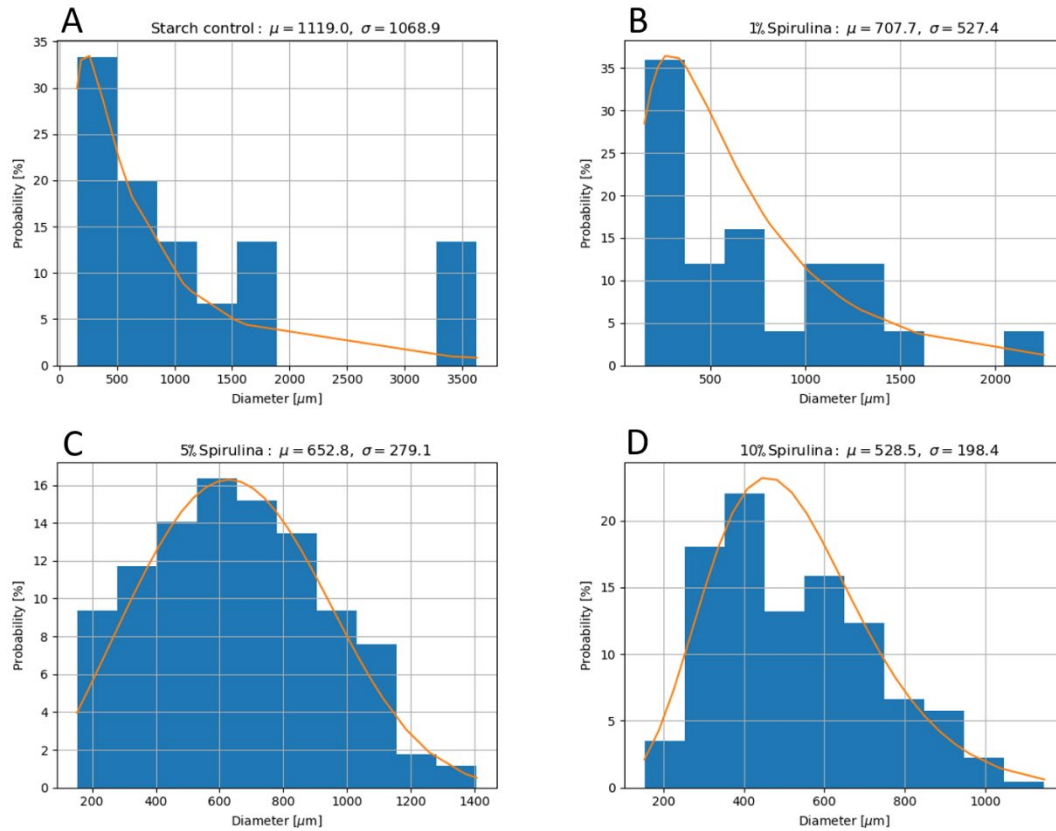
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982

983 **Figure S3.** Test for finding the representative volume of interest (RVI-test), based on the  
 984 Conn. D. as the control parameter. The optimal height of the VOI was chosen at 11.2 mm  
 985 and 7.1 mm for the starch and hybrid starch-*Spirulina* foams, respectively.

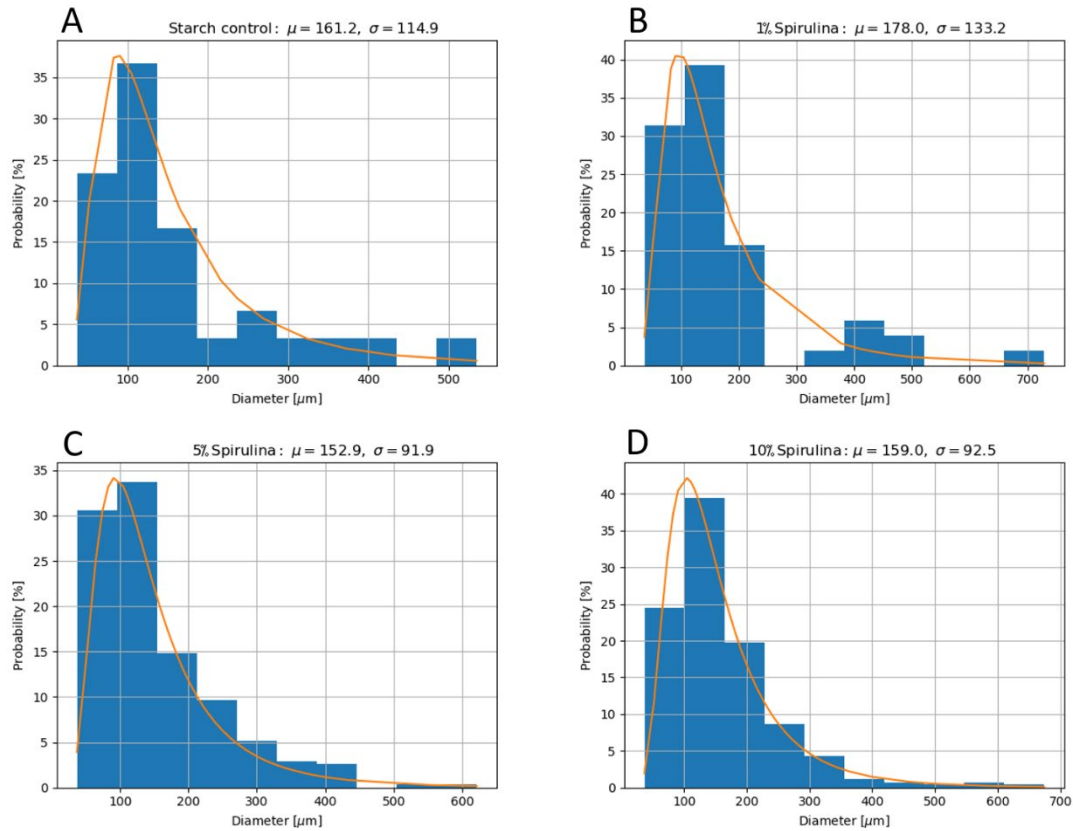
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988 **Figure S4.** Pore size distribution of the extruded foams (A) pure starch, (B) 1% *Spirulina*,  
 989 (C) 5% *Spirulina* and (D) 10% *Spirulina*, as quantified by Method 1. A Weibull distribution  
 990 (orange) was used to fit the data. The fitting works rather accurately for continuous pore  
 991 sizes up to 1400  $\mu\text{m}$  as in (C-D) but fails at pore sizes bigger than 2000  $\mu\text{m}$  as in (A-B), due  
 992 to the discrete number of available pores.

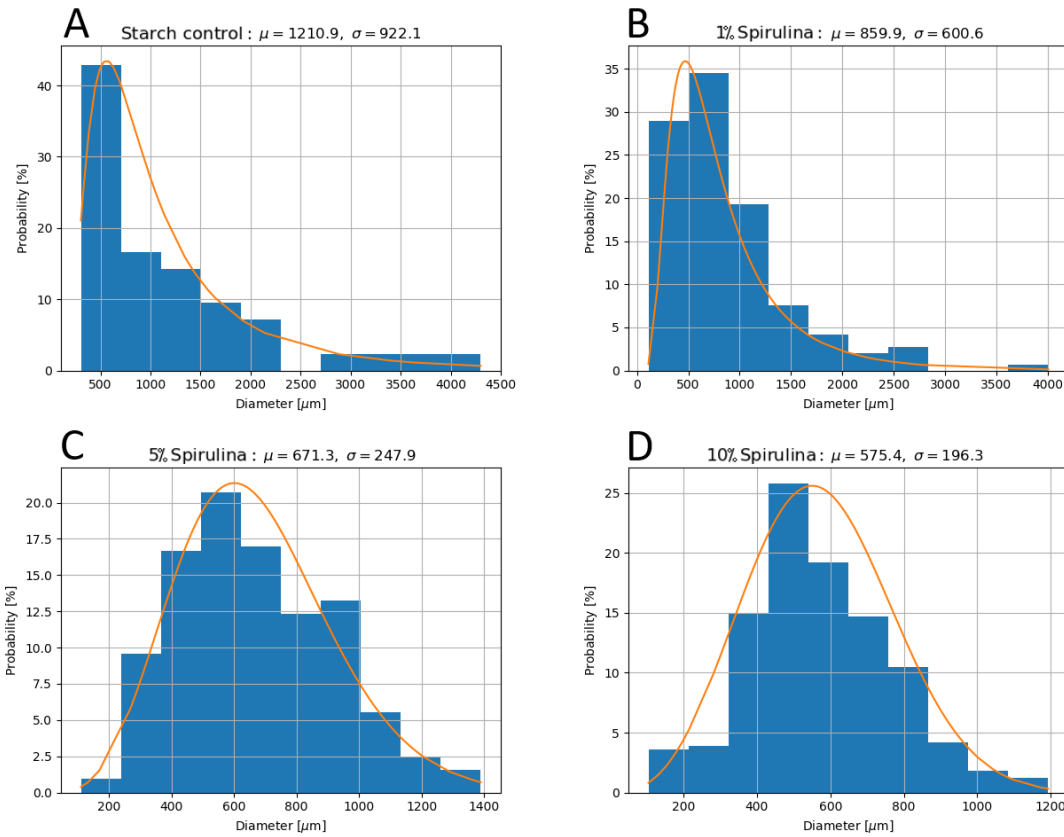
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994

995 **Figure S5.** Throat size distribution of of the extruded foams (A) pure starch, (B) 1%  
 996 *Spirulina*, (C) 5% *Spirulina* and (D) 10% *Spirulina*, as quantified by Method 1. A Weibull  
 997 distribution (orange) was used to fit the data. The fitting works rather accurately for  
 998 continuous throat sizes in (C-D) and less well for the discrete throats sizes in (A-B)

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**Figure S6.** Pore size distribution of the extruded foams (A) pure starch, (B) 1% *Spirulina*,

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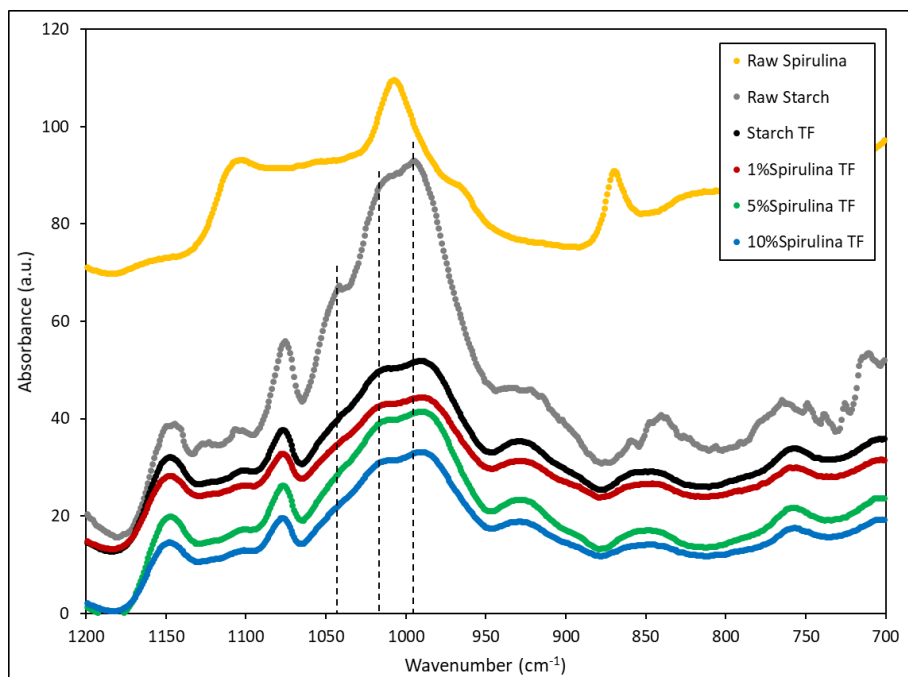
(C) 5% *Spirulina* and (D) 10% *Spirulina*, as quantified by Method 2. A Weibull distribution

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(orange) was used to fit the data. The fitting works rather accurately for all pore sizes (A-D).

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**Figure S7.** ATR-FTIR spectra from the raw starch and *Spirulina* and from the foams after storage. Curves have been offset for clarity.