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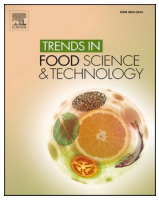

Limitations of recent cellulose studies: How overzealous dispersion technique, fluorescence labeling and interpretation limit the findings of recent safety assessments for cellulose materials

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A B S T R A C T

Microfibrillated, nanofibrillated, and nanocrystalline cellulose materials are next-generation biobased cellulose materials with applications in the food industry as food contact materials and functional ingredients. Manufacturers have adopted a proactive approach to demonstrate the safety of these cellulose materials as they contribute toward a more sustainable economy. Animal studies and alternative testing approaches are needed to fill data gaps in the literature for the physicochemical and toxicological characterization of cellulose materials. Recently, the European Food Safety Authority (EFSA) funded a project that used New Approach Methodologies (NAMs) to assess the hazards of nanofibers, including methods to evaluate simulated gastrointestinal digestion, cellular uptake, local effects via simulated oral exposure, and gut-on-a-chip toxicity models. Upon review, we identified methodological and analytical limitations in these studies, which may affect the study conclusions that oral exposure to cellulose materials is associated with adverse effects. The aim of this Short Communication is to discuss the limitations of the reported findings in the NAM study and provide a balanced perspective of the study and of the safety of cellulose materials. This Short Communication highlights a major concern that the NAM study was published in the public domain without any peer review from cellulose experts. We identify and discuss several major limitations, including an unrealistic dispersion method, lack of controls in fluorescent labeling, lack of proper control groups in the experimental design, incomplete reporting of statistical analyses, bias toward reporting adverse effects, and inconsistent explanations of discordant results within the greater scientific literature.

1. Introduction

Cellulose is the most abundant natural biopolymer on earth. Plant-based microfibrillated and nanofibrillated celluloses (MFC and NFC), as well as cellulose nanocrystals (CNC), are members of a class of renewable and biodegradable materials with the potential to replace petroleum-based materials. These substances exhibit enhanced properties for packaging, including higher strength and better barrier properties (Fotie et al., 2023; Osong et al., 2016). Micro and nanofibrillated celluloses exhibit physical and chemical properties that may be beneficial for various applications as food additives, including their use as a: (i) rheology modifier, (ii) stabilizer, (iii) low-calorie substitute, (iv) fiber supplement, (v) component to improve food quality and (vi) processing aid (Ede et al., 2020; Osong et al., 2016; Wüstenberg, 2014). Additionally, fibrillated cellulose may be used in food coatings to preserve the aroma, texture, firmness, and bioactive compounds of processed fruits (Jung et al., 2018). Applications of fibrillated cellulose further extend to its use as a gelling agent, thermal stability agent, flavor preservative, extrusion aid, and fat replacement (Wüstenberg, 2014). Due to its ability to form stable emulsions, fibrillated cellulose can replace other ingredients in mayonnaise (Heggset et al., 2020; Lee et al., 2022), dairy products (Velásquez-Cock et al., 2019; Yu et al., 2021), and meat products (Wang et al., 2018).

In recent years, many academic, governmental, and industrial stakeholders have committed to the responsible and safe development of next-generation sustainable cellulose materials to enhance the green

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economy. However, some challenges remain in realizing the beneficial effects of next-generation cellulose materials as food packaging and food additive ingredients. The first challenge is the complex regulatory landscape for the approval of cellulose materials for food packaging and food additive uses. As organic carbon-based materials that are not spherical, there is a lack of standardized testing and approaches to characterize these materials physically, chemically and toxicologically as part of a safety assessment. Significant efforts to develop and validate safety testing approaches remain challenging. Because cellulose is derived from nature and is an abundant natural material that has been in the food supply as an ingredient for a very long time (it was grandfathered by the US Food and Drug Administration as Generally Recognized As Safe in 1958), many forms lack the rigor of current food safety testing methods, such as consideration of impacts on the gut microbiome. The main differences with the forms discussed herein are the manufacturing approaches used to produce them.

The European Food Safety Authority (EFSA) recently funded a project to develop and use New Approach Methodologies (NAMs) to assess the hazards associated with oral exposure to several cellulose materials. This Short Communication outlines some significant limitations with the findings and conclusions of two reports from the "EFSA Pilot Project on NAMs for the Hazard Assessment of Nanofibers" (Italiani et al., 2023; Vincentini et al., 2023). We identified several issues with the design and reporting of these studies and raise concern that incorrect interpretations and conclusions were drawn. The authors of this Short Communication represent academic, governmental, and industrial stakeholders with many years of experience working with micro and nanofibrillated celluloses and are publishing this short communication to provide a critical perspective on the limitations of the reported findings.

2. Overview of the study

The EFSA funded the Instituto Superiore di Sanità (ISS; Italian National Institute of Health), the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Sciensano, and the National Research Institute for Agriculture, Food and the Environment (INRAE) to use NAMs to assess the hazards of novel cellulose materials, such as MFC, NFC, and CNC. This project was divided into two related studies: 1) oral exposure: simulated gastrointestinal digestion, uptake, and local effects; and 2) exploring the use of gut-on-a-chip models for risk assessments. The goal of Part 1 was to evaluate potential hazards and mechanisms of effect of micro and nanocelluloses through oral exposure using NAM-based testing. Part 2 focuses on assessing the biological fate and effects of digested micro and nanocellulose in a human gastrointestinal tract (GIT) model using both classical *in vitro* models and novel complex 3D *in vitro* models.

The first study used a tiered approach to evaluate oral exposure hazards of different forms of cellulose. In Tier 1 testing, nine materials were tested to obtain information on physicochemical properties and toxicity, including: two MFC [Valida S231 (Sappi) and High fines slurry (University of Maine-PDC)]; NFC TEMPO-oxidized cellulose nanofiber (TOCN; UMaine-PDC); three CNC [NG01NC0102 (Nanografi), NCV100-NAL90 (CelluForce) and CNC-PDC (UMaine-PDC)]; two bacterial nanocellulose (BNC) [BNC-01-AU (Nata de coco; Cass Materials Pty Ltd, Perth, Australia) and BNC-02-PT]; as well as microcrystalline cellulose (MCC). Note that we refer to Valida S231 and High fines slurry as MFC because these materials have lengths of ten to hundreds of microns, while the EFSA NAM study referred to them as nanofibrillated cellulose. All the endpoints and materials tested in Tiers 1, 2, and 3 are described in Table 1. The second study evaluated pristine MFC (Sappi Valida S231C) and CNC (CelluForce NCV100-NAL90) digested by simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF). The potential effects of cellulose exposure in the GIT were evaluated with several *in vitro* models, summarized in Table 2.

Table 1
Endpoints tested in each tier of the study in Part 1.

Tier	Material	Endpoints	
Tier 1	Valida S231, High fines slurry, TOCN, NG01NC0102, NCV100-NAL90, CNC-PDC, BNC-01-AU, BNC-02-PT, MCC	Physicochemical characterization	Zeta potential, size (TEM), inorganic contaminants, crystalline structure, macromolecular chemistry, functional groups
		High content analysis	Direct cell count, nuclear size, nuclear intensity, apoptosis (active Caspase-3), pro-inflammatory response (NF-κB & IL-8), DNA damage (γH2AX & pATM S1981), oxidative stress (DCFDA)
Tier 2	Valida S231 and NCV100-NAL90	Physicochemical characterization	Number-based size and shape distribution of pristine and fermented particles
		Digestion and degradation	
	Valida S231, CNC-PDC and BNC-02-PT	High content analysis	Same as Tier 1
		Microbiota modifications	
		Cellular uptake	
		Barrier function impairment	TEER, LY translocation
		Genotoxicity	Comet assay, Fpg-modified comet assay, Phospho Histone H3 (PH3) test
		Mucus production and secretion	
Tier 3	CNC-PDC	Barrier function impairment	TEER, LY translocation
		Cellular uptake	
		Translocation	

Table 2
In vitro models and endpoints in each model used in the study of Part 2 to test Sappi Valida S231C and CelluForce NCV100-NAL90.

In vitro model	Endpoints
N/A	Physicochemical characterization: Morphology (TEM), crystallinity (Raman analysis)
Caco-2 cell	Barrier integrity (TEER), immunohistochemistry (ZO-1, *E*-cadherin and Villin), inflammation (IL-6 and IL-8 cytokine production)
Human primary organotypic 3D small intestinal tissue model (EPIINTESTINAL™)	Barrier integrity (TEER), viability (MTT assay), cytotoxicity (LDH assay), immunohistochemistry (CK-19, ZO-1 and Villin), inflammation (IL-6 and IL-8 production and gene expression)
3D-Intestinal Organoids (IHOS)	Growth, morphology, cytotoxicity (LDH assay), gene expression of LGR5 and ZO-1
Primary human macrophages	IL-6 production
Co-culture of Caco-2 and human primary monocyte-derived macrophages	Barrier integrity (TEER), IL-6 and IL-8 production
Immunocompetent 3D perfused "GUT–ON–A-CHIP" model	IL-6 and IL-8 production, barrier integrity (TEER), cytotoxicity (LDH assay)
Human intestinal epithelia and primary macrophages	Cellular uptake (TEM and confocal microscopy)

3. Study limitations

3.1. physicochemical characterization

The NAMs report stated that many existing studies on the characterization and toxicity of cellulose materials lack suitable dispersion protocols to deagglomerate the materials appropriately and that the test conditions in these studies do not represent a worst-case scenario. Therefore, the authors used a probe ultrasound sonicator with approximately 7 kJ energy to disperse the stock suspensions of cellulose materials and developed protocols to maintain the stability and the degree of deagglomeration of the stock and diluted suspensions (Vincentini et al., 2023). The result of the NAM study and existing literature demonstrate that sonication fundamentally changes the physical characteristics of cellulose materials, which can impact how these materials behave in biological testing.

Sonication is one of the techniques suggested by the Joint Research Centre (JRC) Guidance to reduce agglomeration/aggregation and studies have shown that sonication is useful to disperse individual fibers before microscopy imaging and biological exposure in the case of nanocellulose (Mulenos et al., 2019, 2021); however, the guidance also emphasizes that the power should not be too high to break the constituent particles (Hubert et al., 2023). According to the JRC Guidance (Taurozzi et al., 2012) energy density (W s/mL), the amount of delivered energy per unit of suspension volume plays a key role in the effectiveness of the sonication process and therefore, a validation study is required to optimize input power, time and suspension volume to achieve the desired degree of dispersion and minimize unwanted side effects. However, the authors did not specify whether they followed the Nanogenotox protocol (Keld Alstrup Jensen, 2011) using the highest power 400 W as energy input and failed to explain any validation or the rationale for using 7 kJ of energy. Without any validation study, the authors cannot conclude that sonication properly dispersed fibers without damaging or/and further refining fibers to smaller particles and altering actual size distribution (see detailed discussion below). In the Part 1 report, the authors conducted visual inspection to demonstrate that sonication did not destroy the individual fibers and concluded that only BNC-02-PT material had damaged fibers. However, it is not objectively clear that visual inspection is adequate to distinguish between deagglomeration/disaggregation and the actual breaking of the fibers. Using sonication to completely disperse cellulose materials to represent the “worst-case scenario” is the core argument of the NAM study. However, TEM image analysis for cellulose materials requires comprehensive image assessment, measuring hundreds of particles over many TEM images. Visual inspection of differences in TEM images without any objectively measured features and criteria for differences or change in size is qualitative at best and subjective and biased. We recommend the NAM study authors consider possible modifications at different length scales and use additional techniques to span the length scale more effectively. Further, the authors point out that micrometer-sized MCCs were broken down to nanometer-sized fibers after sonication. Sonication of pulp has been demonstrated as an effective pre-processing treatment in the production of CNF, and CNC to facilitate the deconstruction of the starting pulp material (Teo & Wahab, 2020). In addition, sonication of MCC has been used to produce CNC-like materials (Mohd Amin et al., 2015). Therefore, it is unsurprising that MCC was refined to nano-scale fibers after sonication in the NAM study. The MCC after sonication appears to be refined to the point that adverse effects were reported, despite decades of safe use and evidence on the safety of MCC (see more detailed discussion in Section 2 below). These results show that the sonication energy was high enough to refine the cellulose materials, generating nano-scale fragments of the substances. Although sonication is one of the dispersion methods used for cellulose nanomaterials (Chanda & Bajwa, 2021), this is not representative of the commercial forms of the MCC and MFC and cannot be considered a realistic worst-case scenario for toxicity testing as it destroys the starting material. Common commercially relevant approaches to disperse cellulose materials depends on the production method and end-use application including pulpers, agitators, and mixers with significantly lower energy input than sonication.

Furthermore, JRC guidance recommends precautions to prevent the probe tip from introducing contaminants, such as metal, during the sonication process. Studies have shown that suspensions can be contaminated with metal particles from tip erosion during the sonication process (Betts et al., 2013; Hutin & Carvalho, 2022; Pradhan et al., 2016). The authors measured inorganic contaminants in all samples before sonication. However, they did not measure inorganic contaminants after sonication, so it is unclear if sonication introduced metal contaminants that could be the source of the reported observed adverse effects in the *in vitro* studies. Therefore, the sonication process used to disperse tested cellulose materials is not an appropriate approach for dispersing these fibrous materials for safety assessment as it can change particle morphology/size, introduce contamination, and is not representative of commercial dispersing approaches.

The authors stated that the physicochemical characterization and descriptive TEM analysis were performed according to the EFSA Guidance on Nano-Risk Assessment (More et al., 2021). However, this guidance does not include details on measurement methods used for material screening. The guidance describes the definition of nanomaterials and lists the required information from the TEM results. The guidance recommends reporting on median particle size, number-based size distribution, and an estimate of the uncertainty and width of the size distribution. However, a complete characterization following the EFSA Guidance on Nano-Risk Assessment was only conducted and reported for Valida S231 and the High fines slurry. At the same time, only the estimated median fiber diameter and length were reported for the other six cellulose materials tested in Tier 1 (Annex-C, see the link to Annex-C in Table S2 in Supplementary information).

In addition, the TEM approach adopted in this report did not fully follow recent JRC Guidance (Hubert et al., 2023). First, the authors did not evaluate the uncertainty of the TEM data to validate their TEM methodology and results. Instead, they estimated the uncertainty of their TEM data by using the TEM results of a different study (Verleysen et al., 2019). Verleysen et al. (2019) validated their TEM method for size measurement of several certified reference nanoparticles (NPs), including colloidal silica, titanium dioxide, cerium oxide and gold nanorods. Colloidal silica NPs and gold nanorods were vortexed for 5 s while TiO_2 NPs and CeO_2 NPs were sonicated following the dispersion protocol from NANoREG (NANoREG, 2014) and Pradhan et al. (2016). The method used by Pradhan employed a 20 % sonication amplitude (continuous mode) for 882 s (Pradhan et al., 2016). The aspect ratios of the tested particles and dispersion protocols differ between the NAM study and the study conducted by Verleysen et al. (2019). Therefore, it is inappropriate to use the uncertainty obtained by Verleysen et al. (2019) to estimate the uncertainty of TEM analysis in the NAM study. Second, the JRC Guidance and standards cited for TEM analysis require sufficient images and particles to be analyzed to reach adequate accuracy (Hubert et al., 2023). However, due to inadequate reporting, it is not clear whether the measurements obtained by the authors meet the requirements of JRC Guidance. Therefore, the reliability of the reported particle size distribution is uncertain, and the conclusion that all tested materials are nanomaterials may not be accurate.

3.2. high content analysis (HCA)

In the Tier 1 test, cytotoxicity endpoints associated with exposure to nine cellulose materials at a concentration range of 0.4 μg/mL to 120 μg/mL for 24 hours were evaluated in undifferentiated intestinal Caco-2 cells and THP-1 macrophages using HCA. All cellulose materials, excluding MCC, had no or slight effects at the highest concentrations tested (120 μg/mL) on the majority of cytotoxicity endpoints assessed, except interleukin 8 (IL-8). Adverse effects were reported for the

sonicated MCC in this study. Exposure to sonicated MCC reduced cell viability and induced pro-inflammatory response marker NF-κB and apoptotic marker active Caspase-3. MCC has been safely consumed for decades and is listed as "Generally Recognized as Safe (GRAS)" on the United States Food and Drug Administration (FDA) Food additive status list (Food and Drug Administration, 2024) and is permitted as an ingredient "*quantum satis*" in Europe (E460) (Maged et al., 2018), meaning it may be used up to a level of technical effect without limitation. Numerous *in vivo* and *in vitro* studies have demonstrated minimal toxicity of MCC at extremely high concentrations, and several scientific expert food and feed committees have considered it unnecessary to set an acceptable dietary intake (ADI) for MCC (Bampidis et al., 2020; Bangar et al., 2023). Furthermore, the NAM study reported that sonicated MCC significantly reduced cell counts (Figure F5 in Annex-F) and induced higher IL-8 secretion in differentiated THP-1 cells treated for 24 hours (Figure F6). In conclusion, the NAM study has shown that the sonication process can fundamentally change the physicochemical properties of the cellulose suspensions, which may alter the cellular response (Foucaud et al., 2007).

Another issue related to the HCA study is that the vehicle/solvent control (Gibco Dulbecco's Modified Eagle Medium and Gibco Roswell Park Memorial Institute 1640 Medium) induced adverse effects. For example, in Caco-2 cell studies, increases in cytotoxicity, apoptotic marker active Caspase-3 and the DNA damage marker γH2AX were observed in some control samples (without micro or nanocellulose) (Annex-L), indicating that the media used in the study can induce adverse effects or potentially may have been contaminated. The study also failed to account for significant variations in the vehicle controls in different groups (Figure F4, Supplementary Figure F4 and Supplementary Figure F7 in Annex-F). While subtle differences in cellulose material treated groups were detected, the vehicle control group induced significantly different responses, suggesting that reported adverse effects are at least partially or entirely due to the solvent/media, and not cellulose exposure.

3.3. Cellular uptake

In Tier 2 testing in Part 1 of the study, cellular uptake of CNC-PDC, Valida S231, and BNC-02-PT in Caco-2, macrophage differentiated THP-1 monocultures and triculture model (Caco-2, HT29-MTX and Raji B cells) after 24- and 72-h treatment was evaluated with a fluorescence-detection technique using confocal laser scanning microscopy (CLSM). The experimental approach used to evaluate potential cellular uptake in this study suffered several issues.

3.3.1. Fluorescent labeling

We found the fluorescent labeling procedure used to detect cellulose had multiple limitations, including whether the observed fluorescence in the experiments (indicators of uptake) was due to the cellulose materials, free label, or label attached to fragmented cellulose molecules. The study report stated that dispersion protocols allowing maximal deagglomeration without altering the size and shape of the constituent particles (*i.e.,* cellulose fibers and crystals) were developed for each study material. However, as previously discussed, the sonication process significantly altered the size and morphology. Sonication often leads to smaller size and higher crystallinity index (Bhat et al., 2023). Studies have suggested that probe sonication at a much lower power can decrease the size of cellulose micro and nanomaterials and change the surface chemistry, rendering them more reactive (Klayya et al., 2021; Rahmadiawan et al., 2023; Redlinger-Pohn et al., 2022; Sharma et al., 2021). Since the Carbohydrate Binding Module (CBM) used in this study bonds specifically to crystalline cellulose, these smaller, artificially created particles will have more labels bound to the surface and greater fluorescence associated with them than the larger, as-received materials.

Calcofluor White (CFW) non-selectively binds to cellulose, chitin, glucans, and their oligomers (Engle et al., 1994; Wu et al., 2008). The CBM used in this study (CBM3a) binds to crystalline (insoluble) cellulose on the hydrophobic surface of the ring (Chundawat et al., 2021; Zhang et al., 2013). For both fluorescent labels, they are bound physically and reversibly. Labeling through physisorption is always in equilibrium with the free label. Further, how the researchers removed the excess labels for micro and nanocellulose is unclear. The experimental protocol in Annex-N stated, "GFP-CBM3 was used at the concentration of 0.05 μg/ml in PBS, incubated 1h with the NC, rinsed with PBS, followed by CLSM imaging." Many researchers use dialysis to remove excess labels, though the static diffusion almost always leaves some free dye loosely bound to the surface of the cellulose (Abitbol et al., 2013; Foster et al., 2018; Huang et al., 2013). Forced dialysis (such as stirred cell nanofiltration or tangential flow filtration) is the preferred method. These binding studies also noted that other studies have shown lateral diffusion of CBM and that slight changes in binding conditions can alter the affinity of CBM towards cellulose. In the Chundawat study, authors found that about 30% of bound CBM3 unbinds upon a buffer wash and that even at the lowest concentrations of CBM3 used to label the cellulose, approximately 20% of CFW remains unbound to cellulose (Chundawat et al., 2021). Therefore, it is unclear how much of the observed signals in the NAM study were from the unbound free label.

3.3.2. Lack of control groups

The report stated that: "to demonstrate that this dye is specific for (nano)cellulose also unexposed cellular controls were stained with CFW" and "unexposed cellular controls were treated with GFP-CBM3 to demonstrate that this dye is specific for (nano)cellulose". However, these images were not included in the report or annex. These images should be used as a control of free label to compare to the labeled cellulose samples and are critical to demonstrate that there was no autofluorescence, free fluorescence label, and non-specific binding associated with CFW or CBM3 in the absence of cellulose.

3.3.3. Inadequate interpretation of confocal microscopy results

In the confocal microscopy study, the authors concluded that cellular internalization of cellulose materials was observed. However, the images provided in Annex-N did not show cellulose materials on the nuclei side of the cell membrane (*i.e.*, internalized). Moreover, only CNC samples overlap with the cell membrane, while other samples were observed on the cell membrane surface. The 3D images in Annex-N are not true 3D reconstructions of the cell. Instead, they are 2D images of the cross-section of 3D images, which was inadequate to confirm whether fluorescence was inside the cells or attached to the membrane surface. In Figure 36 of Part 2 of this study, the confocal images demonstrated that Valida S231C (MFC) and NCV100-NAL90 (CNC) (blue fluorescence labeled) were outside or overlapped with the cell membrane (green fluorescence). What authors have identified in the results and discussion as "uptake" would be more appropriately identified as "associated cellulose" since there is no clear evidence that the cellulose penetrated the cell membrane. Moreover, the authors stated that the internalization rate was increased to 3.3–4.7% at 72 h in the triculture model but did not include any images to support this conclusion. Given the study limitations and the data presented, we do not agree with the finding that there was a conclusive demonstration of cellular uptake of any tested forms of micro/nanocellulose.

3.4. Discordant findings

Several *in vivo* and *in vitro* toxicity studies have demonstrated safe oral consumption of cellulose materials, but the NAM study did not comprehensively discuss their findings in the context that they are discordant with the scientific literature. We have summarized 37 acute, sub-chronic, and chronic oral toxicity studies and genotoxicity studies on MFC and nanocellulose in Table S1 (Supplementary information). The majority of *in vitro* and *in vivo* studies demonstrated no toxicity associated with cellulose materials exposure at a range of concentrations

from very low (14 μg mL^{-1}) in *in vitro* study on *Caenorhabditis elegans* to extremely high (21 wt % and 2000 mg kg^{-1} body weight) in *in vivo* studies. Eight studies used sonication as their dispersion method (Catalán et al., 2015; Catalán et al., 2017; de Lima et al., 2012; Fujita et al., 2022; Lopes et al., 2020; Mackie et al., 2019; Moreira et al., 2009; O'Connor et al., 2014, pp. 225–246). However, only three showed effects associated with cellulose material exposure *ex vivo* and *in vitro*, including absorption of nutrients onto cellulose (Mackie et al., 2019) and induction of DNA damage determined by comet assay (Catalán et al., 2017; de Lima et al., 2012), while no *in vivo* study demonstrated any toxicity caused by cellulose exposure.

The authors suggested that the sub-chronic oral toxicity studies conducted by Ede et al. (2020) on CNC (Ede et al., 2020) and Ong et al. (2020) on MFC (Ong et al., 2020) had significant shortcomings, "use of high concentrations (up to 4% w/w) in the absence of any dispersion protocol ensuring appropriate deagglomeration of the test materials" (Vincentini et al., 2023). However, in these studies, all tested substances were prepared by vortexing, which is the verified dispersion method recommended by manufacturers of cellulose materials for use in food and adopted by their producers, and validation studies were conducted that demonstrated even distribution of cellulose materials throughout the feed used in the studies, in accordance with OECD test guidelines followed. In contrast, sonication is used in the production process of CNC but is not the suggested dispersion method by manufacturers. Therefore, a fundamental difference between the NAM report and the other studies briefly mentioned yet dismissed in the report (*e.g.,* Ede et al. (2020), Pradhan et al. (2020) (Pradhan et al., 2020) and Ong et al. (2020)) is that they vortex to disperse the materials, representing the worst-case scenario in realistic conditions, whereas the sonication used for dispersion in the NAMs study does not reflect realistic conditions of use in food or food packaging.

Moreover, the NAM study did not identify the dispersion technique as an issue in other *in vivo* studies reporting adverse effects associated with cellulose exposure when these studies used "inappropriate" dispersion methods like vortexing or no dispersion (DeLoid et al., 2019; Khare et al., 2020). Furthermore, the *in vivo* studies reported by Ong et al. (2020) and Ede et al. (2020) followed internationally accepted Organisation for Economic Co-operation and Development (OECD) Technical Guidelines (TGs) 407 and 408 according to Good Laboratory Practice in certified labs while the methods used in NAM studies were not conducted under these conditions. We disagree that OECD TGs are not valid for this class of materials.

In the NAM study, cellulose is introduced at low concentrations without the addition of any other digestant. The absence of secondary materials in the *in vitro* model in the NAM study ignores the possibility that MFC and nanocellulose will bind with larger carbohydrates and become less penetrable to the gastrointestinal tract in animals. This may explain why no *in vivo* studies that used sonication as a dispersion method have found any oral toxicity or genotoxicity associated with cellulose exposure, while a few *in vitro* studies have reported toxicity.

3.5. Bias in interpreting and discussing results

The NAM Study report demonstrated a bias toward 'positive' (adverse) results in interpreting and discussing results. When the results demonstrate a statistically significant difference for MFC, NFC, CNC, or BNC, the authors used the phrase "significant"; but when differences were not statistically significant or no statistical analysis was provided, the authors still interpreted differences as 'significant'. We have highlighted an example of this bias in the HCA results above. Another example is the interpretation of the loss of barrier function study in Part 1. The authors concluded that there was only partial recovery in trans-epithelial/transendothelial electrical resistance (TEER) for some materials (*e.g.*, High fines slurry from the University of Maine) after 72 hours; however, TEER measurements reported in Table P2 of Annex-P demonstrated a full recovery for all materials. None of the three substances tested were significantly different compared to control triculture at 72 h. Moreover, the authors stated that the permeability measured by Lucifer Yellow (LY) translocation recovered partially at 72 h for Valida S231, while High fines slurry and BNC-02-PT had reduced permeability compared to the control. However, LY translocation data demonstrated that none of the three cellulose materials tested were significantly different compared to CTR triculture at 72 h (Table P3). Furthermore, the authors suggested that LY translocation from the apical to the basolateral compartment of triculture after repeated exposure to High fines slurry increased compared to the triculture control group, but there was no statistical difference reported in Table P5. Therefore, the data does not support the authors' interpretation of the loss of barrier function findings.

The authors did not include a balanced discussion of their findings, as some non-adverse results were not discussed. For instance, the authors stated that all cellulose materials, except NCV100-NAL90 and BNC-01-AU, significantly increased IL-8 in THP-1 cells (Figure F4). However, most cellulose materials tested, except for High fines slurry, had much lower IL-8 secretion at low and medium concentrations than MCC, which was not discussed. The authors stated that the level of the expressions of JAMA-A and Occludin tended to decrease, but no significant difference was marked in Figure 13 of Part 2. In addition, two different donors were analyzed to determine IL-6 release from macrophages (section 3.3.1 Part 2); however, only the replicates from one donor were used in Figure 24 and the authors concluded that Valida S231 and NCV100-NAL90 exposure-induced measurable effects on IL-6 release without acknowledging the replicates from the other donor nor explaining why these samples were not included.

We have also identified significant bias in how TEM studies are presented and discussed between the two reports in this project and how the results are interpreted with respect to the potential for cellular update. Annex-R of the first report states, "No intracellular MFC fibers (High fines slurry), indicating uptake, were observed" following the Tier 3, repeated exposure of MFC to tri-culture. This is reiterated in the second report with the statement, "In this experiment, Valida S231 (MFC) and NCV100-NAL90 (CNC) were not observed intracellularly (Figure 35). Although the limitations of the analysis were indicated in both instances, the TEM images suggest similar results to the fluorescence images of Part 1 of the study, which we discussed previously and which, based on our review, do not suggest cellular uptake. However, how the TEM images are interpreted and implications for potential cellular uptake vary significantly between the two reports and demonstrate a clear bias. The first paper reports the TEM finding simply as "TEM analyses at Sciensano were unsuccessful owing to methodological issues" and does not discuss the results which suggest a lack of cellular uptake. In contrast, the second report discusses the TEM results and how they suggest a lack of cellular uptake (Italiani et al., 2023; Vincentini et al., 2023). We find the lack of consistency in interpreting significant and non-significant results to be a major shortcoming of these studies.

3.5.1. Uncertainty in statistical analyses

The NAM study neither reported nor discussed the statistical analyses used to determine significant differences, and the authors were not consistent in discussing significant differences observed in certain groups compared to other groups with similar or higher degrees of difference. For example, the authors noted that exposure to NG01NC0102 or Valida S231 at the highest concentration resulted in slight effects on Caco-2 cell counts (Figure F1 in Annex-F), but they did not discuss exposure to lower concentrations of TOCN, which resulted in significantly higher cell counts. Another example includes the dramatic difference in IL-6 and IL-8 induction found between two donors within the same group in most treatment groups and some control groups. However, the authors still emphasized that cellulose materials induced IL-6 and IL-8 secretion (Figure 27). A statement highlighted that, "substantial donor-to-donor variability did not allow to reach statistically significant differences between treatments". Therefore, all the conclusions

before this statement were not based on the data or statistical analysis. Moreover, the authors suggest that Valida S231 exposure increased the production of IL-6 and IL-8 in the basolateral compartment when monocyte-derived macrophages were present. However, this may be true for IL-8 with Valida S231 at 100 μg/mL but not for other groups due to the lack of statistical analysis. Furthermore, it is not clear how the study was able to detect small but significant differences in Figure F4 and Supplementary F4, F6, F15 and F17, but could not detect significance in oxidative stress in Caco-2 cells exposed to MCC at 60 and 120 μg/mL (Supplementary Figure F3) and in the reduction of the pro-inflammatory marker NFkB in Caco-2 cells exposed to High fines slurry, TOCN, Valida S231, MCC and BNC-02-PT (Supplementary Figure F7) when the differences between groups were at a higher degree with similar or lower variation between groups.

Without statistical analyses, it is not possible to evaluate the results. For example, more observations are needed for the HCA studies to compare each endpoint and determine statistical significance among the nine cellulose materials. It is also unclear how differences between groups could be detected given the huge variations in the data. For example, the NAM study report stated that the level of ZO-1 mRNA was higher than the control group after being exposed to Valida S231C (MFC) at 100 μg/mL for 48 hours (Figure 13, Part 2), but the standard deviation of the fold change of ZO-1 gene expression in LPS (positive control) and NCV100-NAL90 (CNC) were the same as their means. Surprisingly, statistical significance was detected when large variations were present within and between groups. The NAM study also drew conclusions from experiments with inadequate sample sizes. For instance, the report states that IL-6 secretion levels were increased in the co-culture model with physical direct contact compared to without direct contact (Figure 27, Part 2). The sample size was two in both Figure 27 and 30, so statistical analysis with the most power would be parametric tests, such as two-sample *t*-test or ANOVA. A two-sample *t*-test is not appropriate for comparing more than two groups, while a one-way ANOVA cannot be used to compare the same group in different panels. To compare the differences among each cellulose treatment, statistical analysis like two-way ANOVA must be performed to compare the differences with and without physical direct contact. However, they would not be able to detect any significant difference between groups since the variations in each group were large and the sample size was very low.

4. Conclusion

Recently, there has been increased interest in using biobased materials such as cellulose in various applications to replace petroleum-based materials. Microfibrillated cellulose and nanocelluloses are emerging substances in food-related applications, including food packaging and food additives. The NAM study was ambitious in testing numerous endpoints with nine cellulose materials, but there are some significant limitations in the methodological and analytical approaches that limit their findings. This Short Communication highlights a major concern that the NAM study was published in the public domain without any peer review from cellulose experts. Here, we identified and discussed four major limitations in the NAM study methodology that have implications for its findings and broad conclusions.

1) Sonication altered the starting cellulose substances and damaged the fibrils rather than disperse them. Sonication is not representative of industrial approaches to disperse cellulose materials and it ultimately affects the material's morphology and size distribution, and how these materials behave in biological systems. MCC has been thoroughly assessed and safely used in food and animal feed for several decades; however, sonication refined these materials to nanoscale cellulose, which may have induced adverse effects.
2) Several experimental designs either did not run proper control groups or present data from control treatments to demonstrate the difference or lack of difference associated with exposure to the tested materials. For example, the result of cellular uptake using fluorescent labeling is inconclusive because there is no control group to demonstrate how much of the observed fluorescence is due to unbound free label.
3) The NAM studies did not discuss their statistical analyses or consistently discuss results in the context of statistically significant differences. The authors show a clear bias toward interpreting and discussing 'positive' (*i.e.,* adverse) results.
4) The NAM studies consistently reported adverse effects of cellulose material exposure but failed to discuss their findings within the scientific literature and explain discordant results demonstrating the safety of this class of materials.

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.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tifs.2024.104620.

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