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Research review paper

Enhancing single-cell bioconversion efficiency by harnessing nanosecond pulsed electric field processing

Iris Haberkorn [a], Lya Siegenthaler [a], Leandro Buchmann [b], Lukas Neutsch [c], Alexander Mathys [a,*]

[a] ETH Zürich, Laboratory of Sustainable Food Processing, Institute of Food, Nutrition and Health, Schmelzbergstrasse 9, 8092 Zürich, Switzerland
[b] Bühler AG, Gupfenstrasse 5, 9240 Uzwil, Switzerland
[c] ZHAW, Bioprocess Technology Research Group, Grüentalstrasse 14, 8820 Wädenswil, Switzerland

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ABSTRACT

Nanosecond pulsed electric field (nsPEF) processing is gaining momentum as a physical means for single-cell bioconversion efficiency enhancement. The technology allows biomass yields per substrate ($Y_{X/S}$) to be leveraged and poses a viable option for stimulating intracellular compound production. NsPEF processing thus resonates with myriad domains spanning the pharmaceutical and medical sectors, as well as food and feed production. The exact working mechanisms underlying nsPEF-based enhancement of bioconversion efficiency, however, remain elusive, and a better understanding would be pivotal for leveraging process control to broaden the application of nsPEF and scale-up industrial implementation. To bridge this gap, the study provides the electrotechnological and metabolic fundamentals of nsPEF processing in the bio-based domain to enable a critical evaluation of pathways underlying the enhancement of single-cell bioconversion efficiency. Evidence suggests that treating cells during the rapid proliferating and thus the early to mid-exponential state of cellular growth is critical to promoting bioconversion efficiency. A combined effect of transient intracellular and sub-lethal stress induction and effects caused on the plasma membrane level result in an enhancement of cellular bioconversion efficiency. Congruency exists regarding the involvement of transient cytosolic Ca^{2+} hubs in nsPEF treatment responses, as well as that of reactive oxygen species formation culminating in the onset of cellular response pathways. A distinct assignment of single effects and their contributions to enhancing bioconversion efficiency, however, remains challenging. Current applications of nsPEF processing comprise microalgae, bacteria, and yeast biorefineries, but these endeavors are in their infancies with limitations associated with a lack of understanding of the underlying treatment mechanisms, an incomplete reporting, insufficient characterization, and control of processing parameters.

The study aids in fostering the upsurge of nsPEF applications in the bio-based domain by providing a basis to gain a better understanding of cellular mechanisms underlying an nsPEF-based enhancement of cellular bioconversion efficiency and suggests best practice guidelines for nsPEF documentation for improved knowledge transfer. Better understanding and reporting of processes parameters and consequently improved process control could foster industrial-scale nsPEF realization and ultimately aid in perpetuating nsPEF applicability within the bio-based domain.

1. Introduction

Imminent challenges connected with world population growth to 9.7 billion people by 2050, as well as anthropogenic causes fostering climate change, are propelling the exploration of novel approaches that offer more sustainable value-chains for energy and food supply and maintain

Abbreviations: CREB, cAMP response element-binding protein; DAG, Diacylglycerol; DNA, Deoxyribonucleic acid; ER, Endoplasmic reticulum; GFR, Growth factor receptors; IP_3, Inositol trisphosphate; JNK, c-Jun N-terminal kinase; MAPK, Mitogen-activated protein kinase; MOSFET, Metal oxide silicon field-effect transistor; MSCs, Mesenchymal stem cells; PEF, Pulsed electric field; PI, Phosphatidyl inositol; PIP_2, Phosphatidylinositol-4,5-bisphosphate; PKC, Protein kinase-c; PRF, Pulse repetition frequency; RNS, Reactive nitrogen species; ROS, Reactive oxygen species.

* Corresponding author.
E-mail addresses: Iris.Haberkorn@hest.ethz.ch (I. Haberkorn), Lya.Siegenthaler@hest.ethz.ch (L. Siegenthaler), Leandro.Buchmann@buhlergroup.com (L. Buchmann), neuc@zhaw.ch (L. Neutsch), Alexander.Mathys@hest.ethz.ch (A. Mathys).

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biodiversity (Chaudhary et al., 2018; Willett et al., 2019). To tackle these challenges with a nondestructive and long-term focused strategy, the bio-based domain, including food and feed systems, material, compound, and fuel production, is required to find sustainable solutions by employing novel resources and technological innovations. In this context, renewable bio-based materials relying on single-cell biorefineries, such as those affiliated with yeasts, bacteria, microalgae, and mammalian cells, could offer promising new applications. Single-cell biorefineries not only resonate well with the food and feed sectors but are also gaining momentum in the pharmaceutical and medical domains. Employing a combined method encompassing biotechnology, tissue engineering, synthetic biology, and molecular biology, these novel approaches envision the production of biomass and compounds, such as proteins or lipids, from cellular cultures that would otherwise be derived from traditional agriculture. They also allow for the production of specific, relevant compounds, e.g., insulin or tissue production (Caporgno and Mathys, 2018; Rischer et al., 2020).

Efficiency enhancement remains a main target in the bio-based domain for rendering the value-chains of single-cell biorefineries into economically viable scenarios. For example, the economic viability of microalgae feedstock production remains hampered by low upstream productivities, leading to an increase in total biomass production costs (Enzing et al., 2014). On the one hand, approaches relying on genetic or metabolic engineering, such as the targeted modification of metabolic pathways of an organism using recombinant DNA technology, have created a foothold for scientific discovery to foster compound or biomass production (Yang et al., 2007) . However, genetic selection or synthetic biology to simultaneously optimize several functional traits of an organism to elevate its biomass productivity are limited. Additionally, consumer acceptance may hinder the incorporation of genetically modified organisms in the food or nutraceuticals domain, and regulatory approval is handled cautiously (Costa-Font et al., 2008). On the other hand, novel technological approaches can aid in advancing single-cell-based biorefineries and are major drivers for improving supply-chain efficiency (FAO, 2017).

In this context, nanosecond pulsed electric field (nsPEF) processing has emerged as a promising technology-driven, resource-efficient innovation for economizing the bioconversion and thus value-chain efficiency of single-cell biorefineries. NsPEF is presumed to mainly act on intracellular structures inducing abiotic, sublethal stress, which was shown to enhance the bioconversion efficiency in prokaryotic and eukaryotic single-cells, including *Arthrospira platensis*, *Chlorella vulgaris*, and *Saccharomyces cerevisiae,* while not affecting the structural and techno-functional properties of desired intracellular components (Buchmann et al., 2019; Buchmann and Mathys, 2019; Haberkorn et al., 2020b, 2019). Unfortunately, the comparison of results and knowledge transfer between different applications of nsPEF in the bio-based domain has up to now been hindered by incomplete reporting and insufficient characterization and control of processing parameters, which need to be addressed to increase the industrial implementation of nsPEF processing. Furthermore, the exact working mechanisms underlying nsPEF-based growth stimulation remain elusive, but a sound mechanistic understanding of the influencing factors would be required to broaden controlled application over several organismal domains and consequently allow for scale-up to industrial implementation. Revealing the mechanisms underlying nsPEF-triggered bioconversion efficiency enhancement would allow fine-tuning of the process and further optimization. Ultimately, nsPEF operation parameters could be adjusted in real time to ensure optimal process performance once the underlying effects are known and can be accurately followed with appropriate online monitoring technology.

To leverage nsPEF implementation for bio-based applications, this study provides an exhaustive, critical review of nsPEF processing with a focus on enhancing bioconversion efficiency in single-cell value-chains. Therefore, the study concatenates (1) the theoretical framework of the electrical engineering principles required for biotechnological applications with (2) cellular response pathways hypothesized to underlie nsPEF-based growth and compound stimulation in bio-based applications to (3) highlight current and future trends of nsPEF processing as an emerging technology in the bio-based domain.

2. Study and review selection criteria

In the first step, studies were included that fulfilled the following criteria: (1) Description of ultrashort electrical pulses within the nanosecond range. (2) NsPEF treatments of plants, microalgae, bacteria, fungi, and mammalian cells. (3) Treatment effects uncoupled from growth stimulation, as well as those targeting growth stimulation, were considered. The first screening yielded 61 studies. In a second step, those 61 studies were screened for the following criteria, while a no-match resulted in their exclusion: (1) Sufficient method and technical-level process documentation, including a complete description of the experimental setup (treatment volume or treatment chamber description, pulse generator, flow field, and others). (2) Indications of the pulse width and electric field strength corresponding to each treated subject were considered minimum information required for inclusion in the review to allow for an adequate comparison. Differences in the quality of documentation of technical process parameters, i.e., a lack of process parameter reporting and established standards for process parameter description, often resulted in incomplete study description and hampered comparability. Therefore, the study screening and reporting focused on the indication of pulse width and electric field strength as a common denominator reported in all studies considered for the review. NsPEF treatments, either conducted as batch or continuous processes, were considered for the study. The second sorting step yielded 28 studies that were considered for the review (Table 1). These 28 studies showed treatment effects with possible relevance for metabolic pathways in relation to the growth process of organisms of different origins, including mammalian cells, bacteria, yeast, microalgae, and plants. Although not fulfilling the criterion of being in the single-cell domain, plant studies were also considered owing to the similarity in cell structure to microalgal cells.

3. Best practice guidelines for nsPEF documentation

Although various studies describe the use of nsPEF in biotechnological or biological applications, the comparability between studies remains limited. Reasons include differences in the studied organisms (e.g., biological taxonomy, size, sensitivity) and study focus on one hand, which are exacerbated by the experimental setup (e.g., batch vs. continuous nsPEF treatment, pulse generators, specific energy input), and gaps in process parameter reporting on the other hand. For example, medium conductivity is a major contributor affecting the treatment outcome but was unreported in 20 of the 28 studies considered for the review. In addition, 6 of the 28 studies did not or did not sufficiently report the number of pulses applied to the target cells during treatment, and if so, indication of the time interval between multiple pulses was missing.

Establishing best practice guidelines for nsPEF process parameter reporting, analogous to those described for conventional PEF processing, could aid in overcoming the limitation of impaired study comparability due to incomplete reporting and thereby leverage the industrial realization of nsPEF. As an attempt to foster complete process reporting and provide a guideline, Table 2 summarizes process parameters, which are recommended to be reported for studies harnessing nsPEF to facilitate knowledge transfer and minimize ambiguity in data comparison. Established process understanding and documentation remain crucial in continuous nsPEF treatment since the influence of dynamic processes such as the flow field or flow rate must be addressed.

Table 1
Studies selected for describing cellular responses underlying nsPEF-based growth stimulation ($n = 28$), including the number of studies reporting pulse width τ_P (ns), electrical field strength E (kV cm^{-1}), and number of pulses n (−).

Target cell	nsPEF parameters			Viability	Effect	Source
	τ_P (ns)	E (kV cm^{-1})	n (−)		Categorized effects measured in the corresponding study	
Cytoskeleton						
Tobacco BY-2	10	33	1	=	Microtubule disorder, actin dissolution, disintegration of nuclear envelope	(Berghöfer et al., 2009)
Tobacco BY-2	10	30	1	+	Cytoskeletal actin dissolution	(Frey et al., 2011)
Tobacco BY-2	100	10	10	–	Delayed premitotic nuclear positioning	(Kühn et al., 2013)
Jurkat	60	15	1	–	Change in cytoskeleton, irreversible speckled appearance of actin filaments	(Stacey et al., 2011)
HeLa	60	15	1	=	Speckled appearance of actin filaments, indication of cytoskeleton breakdown	(Stacey et al., 2011)
Calcium						
Jurkat	60	25, 50	1, 2	NE	Intracellular Ca^{2+} increase, from intracellular stores	(Scarlett et al., 2009)
Jurkat	60	100	1, 2	NE	Intracellular Ca^{2+} increase, from intracellular and external stores	(Scarlett et al., 2009)
Jurkat	10, 30	25	1, 10	NE	No signs of electroporation, increase in intracellular Ca^{2+} concentration	(Vernier et al., 2003)
CHO	300	3.7, 4.5	1, 2	NE	Intracellular Ca^{2+} increase	(Semenov et al., 2018)
CHO	300	11.1	2	NE	Intracellular Ca^{2+} increase, from intracellular stores	(Semenov et al., 2018)
CHO	60	11.2	NE	NE	Intracellular Ca^{2+} increase, from external stores	(Semenov et al., 2013a)
CHO	60	18.7, 22.4, 26.8, 30	NE	NE	Intracellular Ca^{2+} increase, release from ER and Ca^{2+}-induced Ca^{2+}-release	(Semenov et al., 2013a)
CHO	10	112, 168, 206, 243	NE	NE	Intracellular Ca^{2+} increase, from intracellular stores	(Semenov et al., 2013b)
HL-60	60	60	1	NE	Intracellular Ca^{2+} increase, influx of extracellular Ca^{2+}	(Buescher et al., 2004)
HL-60	300	15, 30, 60	1	NE	Intracellular Ca^{2+} increase, from intracellular and external stores	(Buescher et al., 2004)
Neutrophils	60	15, 30, 45, 60	1	NE	Intracellular Ca^{2+} increase, influx of extracellular Ca^{2+}	(Buescher et al., 2004)
Neutrophils	300	10	1	NE	Intracellular Ca^{2+} increase, from intracellular and external stores	(Buescher et al., 2004)
MG63	60	10	10	NE	Intracellular Ca^{2+} increase, from intracellular stores	(Zhou et al., 2018)
ROS/RNS						
C. reinhardtii	25, 50	40	18.8	–	Intracellular oxidative burst	(Bai et al., 2017)
CHO	300	14.4	1	NE	Lipid peroxidation	(Michel et al., 2020)
BxPC-3	100	30	5	+	Intracellular Ca^{2+} increase, ROS increase, DNA fragmentation, caspase activation	(Nuccitelli et al., 2013)
Metabolism & Genes						
A. platensis	100	10	2.05 ± 0.54	+	Overexpression of Na^{+}/Ca^{2+} exchanger/integrin-beta4 and elongation factor Tu	(Buchmann et al., 2019)
S. avermitilis	100	5, 10	20	=	Increase in avermectin production	(Guo et al., 2016)
S. avermitilis	100	15	20	+	Increase in avermectin production, increase in transcription level of aveR and malE	(Guo et al., 2016)
Jurkat	10 60 300	150 60 26	NE NE NE	NE	Caspase activation	(Beebe et al., 2002)
Jurkat	60 300	60 26	1, 10 1, 10	NE	Reduction of kinase activity	(Beebe et al., 2002)
CHO	600	16.2	1	NE	PI signaling pathway	(Tolstykh et al., 2013)
CHO	600 484 60 600	8 16.2 16.2 16.2	NE NE 20 1, 20	NE	Depletion and/or hydrolysis of the PIP_2 from the plasma membrane	(Tolstykh et al., 2017)
HeLa S3	80	20	20, 30	=	eIF2α phosphorylation, translational suppression, activation of PEFK and GCN2	(Morotomi-Yano et al., 2012)
HeLa S3	80	20	40, 60	–	Reduction of eIF2α phosphorylation	(Morotomi-Yano et al., 2012)
HeLa S3	80	25, 30	20	–	eIF2α phosphorylation	(Morotomi-Yano et al., 2012)
MSCs	10 100	20 10	5 5	–	Increased chondrogenic differentiation, upregulation of cartilaginous gene expression (JNK/CREB-STAT3)	(Ning et al., 2019)
Chondrocytes	100	10, 20	5	+	Decrease of GAG production, decrease of COL II and Sox9 gene expression, increase in COL I and COL X gene expression, activation of wnt/β-catenin signaling pathway	(Zhang et al., 2015)

(*continued on next page*)

Table 1 (*continued*)

Target cell	nsPEF parameters			Viability	Effect	Source
	τ_P (ns)	E (kV cm^{-1})	n (−)		Categorized effects measured in the corresponding study	
HCT116 (p53−/−)	300	26	1, 3, 5	NE	Caspase activation	(Hall et al., 2005)
E4 squamous	300	32	10	NE	Caspase activation	(Ren and Beebe, 2011)
E4 squamous	300	42	10	NE	Cytochrome c release	(Ren and Beebe, 2011)
Growth						
*C. vulgaris**	100	10	3.04	+	Increase in biomass yield per substrate ($Y_{X/S}$)	(Haberkorn et al., 2019)
*C. vulgaris**	100	10	4.26	+	Increase in biomass yield per substrate ($Y_{X/S}$)	(Haberkorn et al., 2020b)
A. thaliana	10 100	5, 10, 20 5	100 10	+	Increase in leaf area	(Frey et al., 2011)
A. thaliana	10 10 10 10	5, 20 50 20 10	100 10, 40 10, 40 40, 100	+	Increase in growth on day 5	(Eing et al., 2009)
Tobacco BY-2	25	10	NE	+	Increase in mitotic index	(Frey et al., 2011)
H. ammodendron	100	10, 20	20	+	Increase in seed germination and radical length of seeds	(Su et al., 2015)
*A. platensis**	100	10	2.05 ± 0.54	+	Increase in biomass yield per substrate ($Y_{X/S}$)	(Buchmann et al., 2019)
S. avermitilis	100	10, 20	20	+	Increase in proliferation of colony forming units	(Guo et al., 2016)
S. avermitilis	100	15	20	+	Increase in OD (450 nm)	(Guo et al., 2016)
H. ulmarius (fungus)	100	5	NE	+	Growth stimulation	(Frey et al., 2011)
Chondrocytes	100	10, 20	5	+	Increase in cell proliferation	(Zhang et al., 2015)

Cell viability increase (+), decrease (−), no effect (=), not examined (NE). (*) Continuous treatment.

4. Theoretical framework underlying nsPEF processing in the bio-based domain

4.1. Pulsed electric field processing

In general, the application of PEF treatments to biological cells follows the principle of electropermeabilization (Pauly et al., 1960; Schoenbach et al., 2004). Resembling a circuit model, biological membranes can be regarded as capacitors with an inherent resting potential of approximately −70 mV under physiological conditions (Buescher and Schoenbach, 2003). This negative resting potential is created by the lower dielectric constant of the membrane compared to the dielectric constants of the internal and external medium, which leads to an accumulation of free charges at the membrane (Kotnik and Miklavčič, 2006). The application of an electric field E (V) (unipolar, rectangular pulses) over a time span of micro- to milliseconds with amplitudes of several hundred volts per centimeter, enhances the accumulation of charged ions at both sides of the membrane (Buescher and Schoenbach, 2003; Kotnik and Miklavčič, 2006). Increasing the electrical field strength above a critical value, E_{crit}, results in the induced voltage superimposing the resting potential of the cell, which induces a transmembrane potential $\Delta\Psi_m$ (V) (Toepfl et al., 2006).

For a spherical cell with radius r_m (m), the transmembrane potential $\Delta\Psi_m$ is proportional to the externally applied electric field strength $E(t)$ (V m^{-1}), the form factor (1.5 for spherical cells), the angle with respect to the electric field direction θ (°), the treatment time t (s), and the membrane charging time τ_m (s) (Eq. 1) (Gehl, 2003; Kotnik and Miklavčič, 2006; Raso et al., 2016).

$$\Delta\Psi_m(t) = \frac{3}{2}E(t)\cdot r_m \cdot cos\,(\theta)\cdot\left(1 - e^{-\frac{t}{\tau_m}}\right) \quad (1)$$

The membrane charging time τ_m can be determined by the cell radius r_m, the membrane capacitance C_m (F), the conductivity of the external medium σ_e (S m^{-1}), and the conductivity of the intracellular fluid σ_i (S m^{-1}) (Eq. 2) (Gehl, 2003).

$$\tau_m = r_m \cdot C_m \cdot \left(\frac{1}{2\sigma_e} + \frac{1}{\sigma_i}\right) \quad (2)$$

The membrane capacitance C_m is a product of the vacuum permittivity ε_0 (F m^{-1}) multiplied by the relative membrane permittivity ε_m (F m^{-1}), divided by the membrane thickness d_m (m) (Eq. 3) (Arnold and Zimmermann, 1982).

$$C_m = \frac{\varepsilon_o \cdot \varepsilon_m}{d_m} \quad (3)$$

In theory, the induced transmembrane potential reduces the energy required for the rearrangement of phospholipids in the membrane, leading to the formation of hydrophilic pores and subsequently increased permeability and conductivity of the membrane (Kotnik et al., 2012). Increasing the electric field strength $E(t)$ generates a greater force per unit area and thereby augments the difference in the induced transmembrane potential. Therefore, PEF treatments can cause reversible and irreversible membrane electropermeabilization, depending on the applied electric field strength $E(t)$.

The electric field strength $E(t)$ describes the intensity of the generated electric field between electrodes during the treatment. To achieve a homogenous electric field, a parallel plate electrode setup is recommended. While other configurations are also feasible, they lack the ability to generate a homogenous electric field. The electric field strength E (V m^{-1}) can be determined by dividing the applied voltage U_H (V) by the distance between the two electrodes d_e (m) (Eq. 4) (Kotnik and Miklavčič, 2006; Raso et al., 2016).

$$E = \frac{U_H}{d_e} \quad (4)$$

Following basic electrotechnological principles initially described by Ohm's law (Eq. 5), a voltage U_H applied to a system can be described in relation to the electrical resistance R (Ω) multiplied by the current I (A) (Eq. 5).

$$U_H = R \cdot I \quad (5)$$

Table 2
Parameter set for nsPEF treatment with technically defined and methodologically comparable standards.

Parameter	Unit	Indices	
Organism			
Organism type	Descriptive	Scientific name	Obligatory
Average cell radius	m	r_m	Obligatory
Conductivity intracellular fluid	S m^{-1}	σ_i	Optional
Membrane charging time	s	τ_m	Optional
Cell shape	Descriptive	–	Optional
Membrane capacitance	F	C_m	Optional
Relative membrane permittivity	F m^{-1}	ϵ_m	Optional
Membrane thickness	m	d_m	Optional
Set-up			
Pulse shape	Bipolar/unipolar (square pulse /exponential decay pulse)		Obligatory
Pulse width	ns	τ_P	Obligatory
Pulse number	–	n	Obligatory
Pulse repetition frequency	Hz	f	Obligatory
Treatment time interval (if multiple treatments)	Time (s, min, h)	–	Obligatory
Pulse generator type	Descriptive	–	Obligatory
Electric field characterization	Descriptive (homogeneous/ inhomogeneous)		Obligatory
Applied voltage	V	U_H	Obligatory
Electrode distance	m	d_e	Obligatory
Electric field strength	V m^{-1}	E	Obligatory
Electrode configuration	Descriptive (Parallel plate-plate, Coaxial)	–	Obligatory
Treatment chamber volume	m^3	V_0	Obligatory
Volumetric flow rate	m^3 s^{-1}	V	Obligatory
Treatment chamber type (geometry, material)	Descriptive	–	Obligatory
Specific energy input	J kg^{-1}	W_s	Obligatory
Treatment medium			
Medium conductivity	S m^{-1}	σ_e	Obligatory
Medium pH	–	pH	Obligatory
Treatment medium composition	Descriptive	–	Obligatory

To reach matched load conditions, the electrode distance has to be adjusted so that its resistance R (Ω) is equal to the pulse generator resistance (Buchmann et al., 2018b). For a plate-plate electrode setup this can be calculated as follows (A (m^2): electrode surface area; σ_e (S m^{-1}): conductivity of the external medium; Eq. 6):

$$R = \frac{1}{\sigma_e} \cdot \frac{d_e}{A} \quad (6)$$

4.2. Electrotechnical principles of nsPEF processing

For nsPEF processing, Ohm's law requires adaptations, as the electrical resistance R is replaced by the frequency-dependent impedance Z (Ω) (Buchmann et al., 2018b) (Eq. 7).

$$U_H = Z \cdot I \quad (7)$$

The impedance in turn can be described by the external medium conductivity σ_e and a system's admittance Y_c (S) (Eq. 8) (Buchmann et al., 2018b).

$$Z = \frac{1}{\sigma_e \cdot \frac{A}{d_e} + Y_c} \quad (8)$$

NsPEF processing applies pulses high in an electric field and short in duration (1–100 ns). The rise time of the pulse and its duration are below the time required to fully charge the plasma membrane, resulting in treatment effects primarily at the intracellular level (Beebe and Schoenbach, 2005). Under those processing conditions, the transmembrane potential of an organelle exceeds that of the plasma membrane. Consequently, predominantly subcellular membranes are targeted, without causing irreversible damage to the plasma membrane (Kotnik and Miklavčič, 2006; Tekle et al., 2005). Spherical biological cells can be regarded as an electric equivalent circuit model where cell and organelle membrane exhibit dielectric characteristics (Fig. 1). Thereby, cell membranes act as capacitors with rather low conductance between the cytoplasm and the extracellular fluid resembling an RC-element assembled of the extracellular fluid (resistor R) and the cell membrane (capacitor C). This structure creates a difference in the electrical potential, which is associated with the membrane potential. Applying an electric field results in the accumulation of charges at the membrane altering its conductance. Whereas pulse durations between micro- and milliseconds primarily target outer membranous structures, applying nanosecond pulses affects inner membranous structures. For PEF applications in the micro- and millisecond domain, the proposed RC-element is charged resulting in reversible or irreversible electropermeabilization while intracellular effects remain negligible owing the insulating behavior of the charged membrane. For pulse durations below the charging time of the RC-element the intracellular membranes are exposed to the electric field resulting in an increased probability of electric field interactions with intracellular structures comparable to those of the plasma membrane (Schoenbach et al., 2004; Yao et al., 2009). Buchmann et al. (2019) showed that the plasma membrane of *A. platensis* remained largely unaffected following nsPEF treatments. Inducing a transmembrane potential reduces the energy required for the rearrangement of phospholipids in the membrane, leading to the formation of hydrophilic pores and subsequently an increased permeability and conductivity of the membrane, which was assumed to also occur on internal membranous structures (Kotnik et al., 2012). Hence, for nsPEF treatments, nanopore formation in intracellular or organelle membranous structures was suggested as a possible consequence. According to the underlying theory of nsPEF-based growth enhancement, transient abiotic, sublethal stress induced at the subcellular level in biological cells then causes enhanced cell proliferation (Buchmann et al., 2019; Buescher and Schoenbach, 2003; Haberkorn et al., 2019; Kotnik and

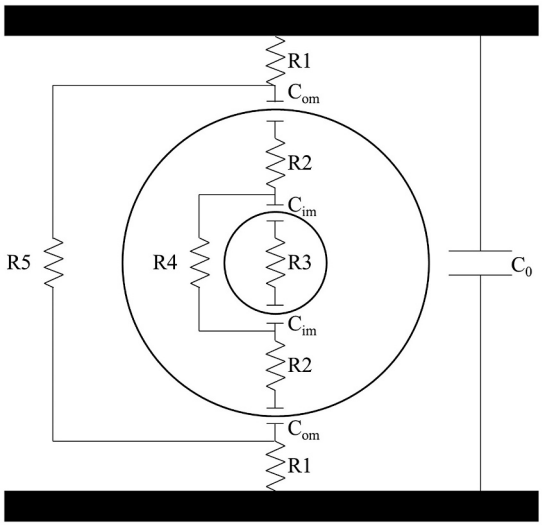

Fig. 1. Model of a biological cell between two electrodes. The cell is displayed as an electric equivalent circuit where cell membranes can be understood as capacitors C and extra (om)- and intracellular (im) fluids as resistors R (Schoenbach et al., 2004).

Miklavčič, 2006). Thereby, the enhancement of growth and the formation of cellular compounds were shown to be possible (Buchmann and Mathys, 2019; Kotnik et al., 2019).

Major process parameters of nsPEF processing comprise the electric field strength *E(t)*, pulse width τ_p, pulse repetition frequency (PRF) *f* (Hz), pulse number *n* (−), pulse shape, and external medium conductivity σ_e. These process parameters relate according to Eq. (9) and affect the specific energy input W_s (J kg^{-1}) applied to a biological cell (Buchmann et al., 2018a, 2018b; Miklavčič, 2017).

$$W_s = E^2 \cdot \tau_p \cdot \sigma_e \cdot n \tag{9}$$

For continuous nsPEF processing, multiplying the PRF by the residence or treatment time *t* (s) determines the pulse number *n*. For continuous treatments, the latter can be determined as the treatment chamber volume V_0 (m^3) divided by the volumetric flow rate $\dot{V}$ ($m^3\ s^{-1}$) given at a specific temperature (Eq. 10).

$$n = f \cdot t = f \cdot \left(\frac{V_0}{\dot{V}}\right) \tag{10}$$

Last, the pulse shape affects nsPEF treatment outcomes, where unipolar/bipolar square wave pulses or unipolar/bipolar exponential pulses can be employed (Fig. 2). Square wave pulses are characterized by their duration, i.e., the time during which the voltage is kept at the maximum or peak voltage and can adequately be controlled and reproduced, the maximum amplitude, and the rise and fall times of the pulse. Buchmann et al. (2018a) define bipolar pulses as two pulses of the same duration but opposite polarities with only a short switch between pulses. The pulse width of exponential decay pulses can be defined as the time required to decrease the voltage to 37% of the peak voltage (Raso et al., 2016; Reberšek et al., 2014). Bioconversion efficiency enhancement as a means of increased biomass yield per substrate ($Y_{X/S}$) was described when applying unipolar square wave pulses of positive polarity (Buchmann et al., 2019; Haberkorn et al., 2020b, 2019).

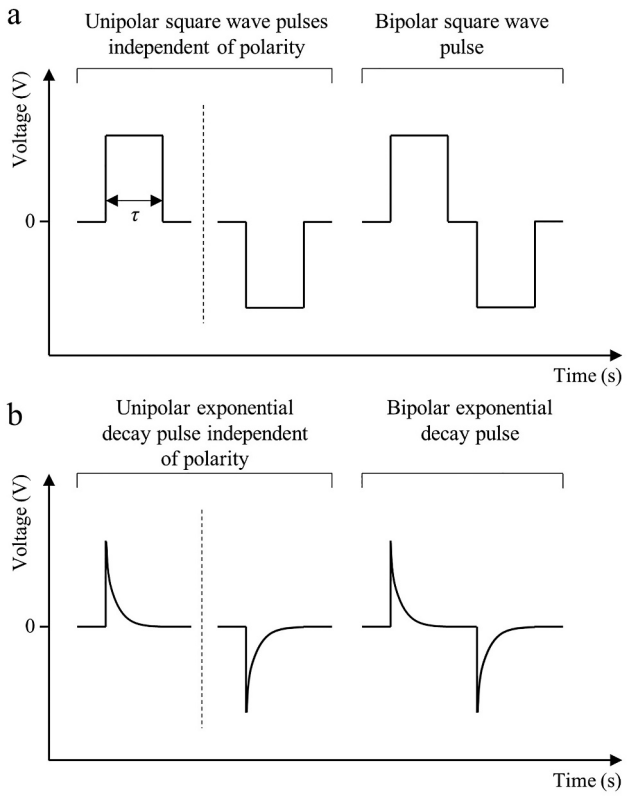

Fig. 2. Model pulses as voltage (V) over time (s) created by an ideal pulse generator. Pulses include (a) unipolar and bipolar square wave pulses with indication of the pulse width τ and (b) unipolar and bipolar exponential decay pulses.

4.3. Generators

The waveforms applied to a cell suspension are enabled by different pulse generator constructions. Nanosecond pulses can be generated by Blumlein generators (Fig. 3a) and diode opening reactors (Fig. 3b). Such high-voltage pulses are characterized by their distinct shape, pulse duration, amplitude, repetition frequency, and number. The energy delivered to the target cells is governed by the pulse number, voltage, current, and duration. The generation of nanosecond pulses requires the utilization of transmission-line generators, which operate through charging and discharging phases. During the charging phase the switch S is turned off and the high-voltage power supply V charges the transmission line T through a resistor R to a given voltage. During the discharging phase the switch S is then turned on and the transmission line T is discharged through the load (impedance Z_L) (Reberšek and Miklavčič, 2010). Accordingly, employing Blumlein generators for nanosecond square pulse formation involves a charging phase where the switch S is turned off and the high-voltage power supply V charges the transmission lines T_1 and T_2 to a given voltage (Fig. 3a). During the discharging phase the switch S is then turned on, which is followed by a discharge of the transmission lines through the load (impedance Z_L) connected to the output (Deng et al., 2001; Kolb et al., 2006; Reberšek and Miklavčič, 2010). Thereby, the load (impedance Z_L) requires an impedance double as large as the impedance of the transmission line to avoid mismatched load conditions resulting in intra-system reflections (Bluhm, 2006). Switching components for pulse-forming networks require a fast turn-on time to form high-voltage square-wave pulses. Metal oxide silicon field-effect transistors (MOSFETs) can achieve such short rise times with a duration of a few nanoseconds and at voltages up to 1 kV. At higher voltages photoconductive switches or spark gaps are preferred over MOSFETs given their short rise time and ability to withstand high voltages (Reberšek et al., 2009). Diode opening switch generators are two-step pulse generators leading to Gaussian-like pulses (Sanders et al., 2009). For charged capacitors, an inductor-capacitor (LC) oscillator begins oscillating with the diode stack forming a pulse on the load itself with a high commutated current and induced voltage on the load. Employing saturable-core inductors instead of air-core inductors enables increasing the commutated current. For manufacturing such pulse generators commonly thyristors or semiconductor-based diodes are used (Blume et al., 2014).

4.4. Impact of nsPEF processing parameters on cells

The impact of the PRF on the treatment outcome of biological cells remains controversial. In general, the PRF determines the amount of electric pulses generated by a pulse generator for one second. Decreasing the time interval between pulses, i.e., increasing the PRF, shortens the recovery time between treatments (Silve et al., 2014). Following the principle of electrosensitization, mammalian cells experiencing electric pulses show increased sensitivity after consecutive treatments. Consequently, treatment efficiency increases with multiple treatments and increasing the PRF. In contrast, the principle of electrodesensitization assumes that a biological cell has to recover to its initial state to enhance treatment efficiency (Silve et al., 2014). Therefore, low-PRF treatments were claimed to show higher efficiency based on the resealing time of the membrane on a timescale of seconds (Jensen et al., 2017; Miklavčič, 2017; Muratori et al., 2016; Silve et al., 2014).

Increasing effects on intracellular structures were reported when shortening the pulse width, and most pronounced effects on organelles were shown for pulse widths between 10 and 100 ns. Increasing pulse widths beyond 100 ns mainly causes effects on plasma membrane levels (Batista Napotnik et al., 2016; Kotnik and Miklavčič, 2006). Intracellular effects were, for example, related to nanopore formation in intracellular,

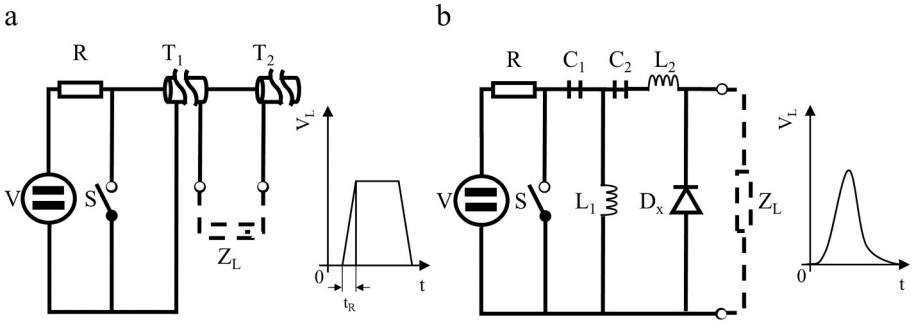

Fig. 3. Electric circuit models and resulting pulse waveform for (a) a Blumlein generator for the generation of square wave pulses and (b) a diode opening switch generator for the generation of pulses similar to a Gaussian function (C: capacitor; D_x: diode; L: inductor; R: resistor; S: switch; T: transmission line; t: time; t_R: rise time; V: high voltage power supply; V_L: amplitude; Z_L: load impedance) (Reberšek and Miklavčič, 2010).

membranous structures, intracellular Ca^{2+} release, and the activation of internal signaling pathways (Fig. 4) (Muratori et al., 2016).

4.5. Bioprocess parameters

An important aspect to consider for successful nsPEF-based bioconversion efficiency enhancement relates to the growth stage and homogeneity of the treated cell suspension. Growth stimulation was shown to depend on the specific growth phase of the treated organism. Significantly enhanced cell proliferation of microalgae (*C. vulgaris*) and the cyanobacterium *A. platensis* was only observed when the treatment was applied in the early exponential growth phase and thus in the rapidly proliferating state of the cells. Similar observations were described for *S. cerevisiae*. For all organism groups, significant growth promotion was observed 5 days following the treatment (Buchmann and Mathys, 2019; Haberkorn et al., 2019; Schoenbach et al., 1997).

NsPEF processing of microbial monocultures ensures treatment application to cells with the same inherent characteristics. Microbial monocultures, however, do not sufficiently reflect the application potential of the technology, as biofilms or other microbial assemblages, such as nonaxenic microalgae cultures, are of great industrial relevance and promising for future bio-based applications. NsPEF treatments

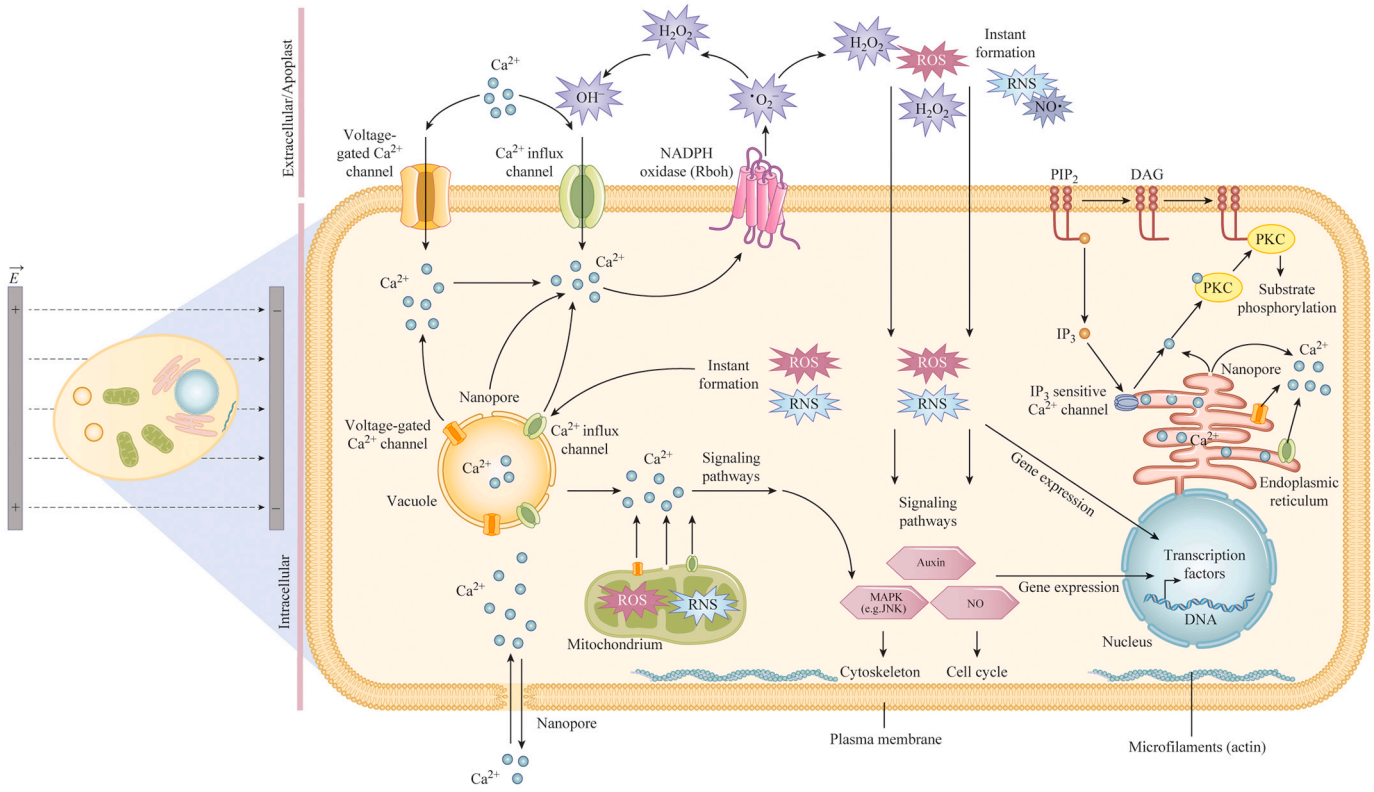

Fig. 4. Treatment responses of biological cells exposed to an electric field during nsPEF processing. Main effects reported relate to nanopore formation on membranous structures and an activation of voltage-gated and Ca^{2+}-influx channels (organelle and plasma membrane), which can result in a mass transfer of molecules across membranes for instance causing transient, cytosolic Ca^{2+} hubs. The formation of reactive oxygen species (ROS), such as superoxide ($\cdot O_2^-$) or hydrogen peroxide (H_2O_2) and nitrogen species (RNS), such as nitric oxide (NO·) was also reported. ROS/RNS formation occurs instantaneously or through the involvement of NADPH oxidase Rboh. ROS, RNS, and Ca^{2+} affect intracellular signaling pathways including those associated with auxin, mitogen-activated protein kinases (MAPK), such as the c-Jun N-terminal kinase (JNK), and nitric oxide (NO) signaling. NsPEF treatments caused Phosphatidylinositol-4,5-bisphosphate (PIP_2) cleavage into Diacylglyerol (DAG) and Inositol trisphosphate (IP_3), resulting in the release of Ca^{2+} from intracellular storage compartments and finally the induction of protein kinase-c (PKC)-dependent signaling pathways. Dissolution of the microfilament (actin) meshwork organization is also shown.

differentially affect prokaryotic and eukaryotic cells due to their distinctively different cell sizes and morphologies. Hence, nsPEF-induced effects might even differ between bacterial species owing to their differences in cell shape and Gram properties. As cells differ in size or shape, they may have different thresholds for the onset of the electroporation effect. In addition, doubling times between organisms might differ in mixed cultures. For example, in nonaxenic microalgae cultures bacteria possess doubling times of hours, whereas microalgae employed in the studies of Haberkorn et al. (2020b, 2019) who investigated growth enhancement of *C. vulgaris* by nsPEF treatments had doubling times of approximately 20 h in the exponential growth phase. Hence, the growth stages of the different population groups in nonaxenic cultures might differ and thereby affect the outcome of nsPEF treatments between microalgae and bacteria in a mixed culture. Thus, nsPEF treatments could enable a selective inducement of intracellular effects on certain microorganisms (Schoenbach et al., 2001).

5. Cellular responses underlying nsPEF-based enhancement of cellular bioconversion efficiency

The exact working mechanisms underlying the enhancement of cellular bioconversion efficiency through nsPEF processing remain largely elusive (in this study bioconversion efficiency relates to the biomass yield per substrate ($Y_{x/s}$) obtained following processing). Studies solely investigating growth stimulation remain scarce and were limited to those reporting about microalgae or plant growth. Translative deliberation based on studies discerning, for example, from the medical domain allows potential protagonists to be narrowed down, although the primary focus of those studies was not the enhancement of cellular bioconversion efficiency. Literature provides striking evidence for different protagonists and their potential orchestration in triggering cellular metabolism towards leveraging bioconversion efficiency, although a distinct segregation of the described effects remains challenging owing to the interplay and harmonization of cellular signaling pathways. For example, congruency exists regarding the involvement of cytosolic Ca^{2+} in nsPEF treatment responses, as well as that of downstream metabolic pathways. In addition, reactive oxygen species (ROS) formation, as well as effects on the cytoskeleton, are described in nsPEF-related literature (Fig. 4).

5.1. Reactive oxygen and nitrogen species

NsPEF treatments may enhance cellular bioconversion efficiency by inducing intra- and extracellular formation of ROS and thereby influence the cellular life cycle. ROS is a collective term describing radical and nonradical molecules, including hydroxyl radicals (·OH), superoxide ($\cdot O_2^-$), and hydrogen peroxide (H_2O_2) (Fig. 4) (Del Río, 2015).

In untreated cells, ROS formation is not only a reaction to external stimuli, but intracellular production of ROS can also be initiated, emanating from mitochondria, the plasma membrane, plastids, and peroxisomes (Bailey-Serres and Mittler, 2006). In plant and animal cells, ROS play an important role in cell signaling and are involved in cell growth, development, responses to external biotic and abiotic stimuli, and programmed cell death. ROS were also shown to influence nuclear envelope dynamics, the organization of the tubulin cytoskeleton, and chromosome movement. They can, for example, induce cell division by triggering mitogen effects or activate mitogen-activated protein kinase (MAPK) cascades, which then influence gene expression (Sauer et al., 2001; Schmidt and Schippers, 2015). A certain level of ROS (especially superoxide; $\cdot O_2^-$) is needed to sufficiently promote plant growth, as they can, for example, activate auxin-dependent cellular growth cycles (Pasternak et al., 2005). Auxin, present in plant cells and microalgae and produced by certain prokaryotes, is in turn responsible for the activation of cell proliferation and differentiation but can also trigger the onset of programmed cell death by initiating auxin-responsive genes at the effector side (Spaepen and Vanderleyden, 2011; Woodward and Bartel, 2005; Zhang and van Duijn, 2014). Similar to plant cells, major sites of ROS formation in animal cells are connected to electron transport chains, such as those located in mitochondria or the endoplasmic reticulum (ER), and they were shown to trigger cell proliferation and differentiation (Sauer et al., 2001). The degree of oxidative signaling is then controlled by spatial and temporal ROS storage and release, regional ROS production, and ROS scavenging (Waszczak et al., 2018).

NsPEF treatments may trigger ROS formation in different ways: (A) direct triggering of NADPH oxidase (respiratory burst oxidase homolog, Rboh); (B) induction of intracellular Ca^{2+} release and subsequent activation of the NADPH oxidase (Rboh), which forms ROS; and (C) instant ROS formation (intra- and extracellular) (Fig. 4).

(A) In *Chlamydomonas reinhardtii*, nsPEF treatments (25 and 50 ns, 40 kV cm^{-1}, 4 Hz) were shown to induce two waves of intracellular oxidative bursts as short- and long-term responses to the treatment (Bai et al., 2017). Oxidative bursts consist of the rapid production of large amounts of ROS in response to external stimuli. Bai et al. (2017) suggested that temporary permeabilization of the plasma membrane induced by nsPEF treatments activated the membrane-located NADPH oxidase (Rboh), which generated superoxide ($\cdot O_2^-$) in the apoplast and triggered a developmental response whose biological consequence was adaptation to (osmotic) stress. NADPH oxidases located in the plasma membrane are major sources of ROS in plant and mammalian cells (Buvelot et al., 1982; Demidchik et al., 2018).

(B) In plant cells, the generation of ROS by NADPH oxidases was reported to stimulate Ca^{2+}-permeable channels located in the plasma membrane, allowing extracellular Ca^{2+} to enter the cell and leading to its transient upsurge in the cytosol, which in turn triggers the activity of Ca^{2+}-dependent NADPH oxidases and thereby increases ROS formation while simultaneously allowing for more Ca^{2+} to enter the cell (Fig. 4). This phenomenon, also denoted as "Ca^{2+}-hub", was shown to be inducible by cytosolic Ca^{2+} following, for example, its vacuolar release (Demidchik et al., 2018). These processes could elevate intracellular Ca^{2+} levels via an influx from the surrounding medium and trigger Ca^{2+}- and ROS-dependent signaling pathways. Literature suggests a strong link between nsPEF treatments and the release of Ca^{2+} from intracellular storage compartments, such as vacuoles or the ER, which, by the activation of NADPH oxidases, could also trigger ROS formation. Nuccitelli et al. (2013) showed that nsPEF (100 ns, 30 kV cm^{-1})-induced ROS formation was dependent on intracellular Ca^{2+} release in human pancreatic cancer cells. However, the applied processing window resulted in cellular apoptosis. Accordingly, the observed response to nsPEF treatment (25 and 50 ns, 40 kV cm^{-1}, 4 Hz) described by Bai et al. (2017) was arrested cell proliferation, increased cell size, and formation of palmella stages (algae aggregation). This suggests that applying a treatment in this process parameter domain initiated a decrease in cell viability, probably owing to the high electric field strengths applied. ROS-induced cell damage, including mitochondrial damage, was shown by Pakhomova et al. (2012) by increasing the number of applied pulses on Jurkat cells but treating at pulse widths of 300 ns (10–100 pulses, 300 ns, 11 kV cm^{-1}, 5 Hz).

(C) Both instantaneous intracellular formation of ROS and ROS formation in the surrounding cell medium were reported to be inducible by nsPEF treatments. Treating cell-free medium with a high number of pulses (3000 pulses, 300 ns, 30 Hz, 1.6 or 4.5 kV cm^{-1}) showed weak formation of ROS (H_2O_2). This slight increase in the extracellular H_2O_2 concentration might contribute to a growth stimulating effect by diffusion-driven activation of intracellular MAPK pathways or aid in increasing cytosolic Ca^{2+} levels by triggering responsive Ca^{2+} channels (Fig. 4). Thus, cells could be stimulated without directly being targeted (Pakhomova

et al., 2012; Sauer et al., 2001). In contrast, Su et al. (2015) detected a decrease in ROS in the buffer system of *Haloxylon ammodendron* seeds but described an increase in reactive nitrogen species (RNS). More precisely, nitric oxide (NO·) was increased following nsPEF treatments (20 pulses, 100 ns, 10–30 kV cm^{-1}). NO belongs to the RNS group, which are key signaling molecules during plant growth (Bailey-Serres and Mittler, 2006). NO is highly diffusible and membrane permeable. Therefore, extracellular NO formation in the buffer system might also act at the intracellular level. The signaling network of NO suggests that under biotic and abiotic stresses, such as those induced by nsPEF treatments, the intracellular NO concentration increases. Consequences include an activation of mitotic processes, stress response genes, defense genes, or cell death. It can further lead to an increase in cytosolic Ca^{2+} concentration, which in turn induces Ca^{2+}-regulated cellular processes (Lamattina et al., 2003).

5.2. Calcium

Calcium is a ubiquitous molecule in plant, mammalian, prokaryotic, and yeast cells, acting at numerous intracellular locations. Cytosolic Ca^{2+} levels are tightly regulated (50–150 nM in plant cells), as the ion is responsible for structural, metabolic, and signal transduction-related mechanisms. An increase in cytosolic Ca^{2+} can occur due to the influx of Ca^{2+} from the extracellular medium across the plasma membrane, where different Ca^{2+} channels and pumps can enable its transport, or through its release from intracellular storage compartments, including the ER, mitochondria, and vacuoles (Fig. 4). On the plasma membrane level, for example, voltage-gated Ca^{2+} channels are responsible for the transport of Ca^{2+}. Hristov et al. (2018) reported that nsPEF treatments of 300 ns pulses (1.8 kV cm^{-1}) raised cytosolic Ca^{2+} concentrations in human embryonic kidney cells. The authors showed that the associated Ca^{2+} hubs in the cytosol were related to an influx through only nanopores on plasma membrane level or through a combination of nanopores and nsPEF-activated voltage-gated Ca^{2+} channels located in the plasma membrane but not to a release from intracellular storage compartments. At the plasma membrane level, the involvement of NADPH oxidases in triggering an increase in cytosolic Ca^{2+} levels might also be plausible (Fig. 4). As elaborated in Section 5.1, Ca^{2+} channel activity was shown to be partially dependent on ROS formation by NADPH oxidases. Stimulated NADPH oxidase activity either through elevated cytosolic Ca^{2+} levels, e.g., through its release from intracellular storage compartments, or through direct triggering could thus contribute to leveraging Ca^{2+} influx and consequently cytosolic Ca^{2+} levels.

The main working mechanism assumed to underlie nsPEF-based bioconversion efficiency enhancement was suggested to be based on the induction of intracellular abiotic, sublethal stress. Kotnik and Miklavčič (2006) showed that pulse widths between 10 and 100 ns mainly act on intracellular structures, including the membranes of intracellular Ca^{2+} storage compartments, such as the ER, mitochondria, and vacuoles (plant cells). Shorter pulse widths increasingly affect intracellular structures rather than causing effects on plasma membrane levels (Hetherington and Brownlee, 2004; Semenov et al., 2013a). Inducing a transmembrane potential upon the application of an electric field reduces the energy required for the rearrangement of phospholipids in the membrane, leading to the formation of hydrophilic pores and subsequently increased permeability and conductivity of the membrane, which in the nanosecond domain also holds true for intracellular organelle membranes (Kotnik et al., 2012). For nsPEF treatments, nanopore formation in intracellular or organelle membranous structures was suggested, which could lead to a release of Ca^{2+} from intracellular storage compartments and thus a cytosolic Ca^{2+} increase (Fig. 4). NsPEF treatments could also act by triggering intracellular voltage-gated Ca^{2+} channels, entailing Ca^{2+} release from intracellular storage compartments (Fig. 4). Such channels were, for example, reported to be located in the vacuole membrane (Demidchik et al., 2018). It was shown that the release of Ca^{2+} from intracellular storage compartments could be controlled by the duration of the pulse stimulus. For example, Semenov et al. (2013b) showed that the pulse width impacted the amount of Ca^{2+} released into the cytosol, where 10 ns pulses recruited significantly more Ca^{2+} from intracellular ER stores than 60 ns pulses.

Accompanied by a rise in cell proliferation of prokaryotic *A. platensis* after repeated continuous nsPEF treatment (9 Hz, 100 ns, 10 kV cm^{-1}), overexpression of two proteins, Na^{+}/Ca^{2+} exchanger/integrin-beta4 and elongation factor Tu, was observed by Buchmann et al. (2019). While the elongation factor Tu is a protein binding tmRNA (transfer-messenger RNA) with a pivotal role in protein biosynthesis, the Na^{+}/Ca^{2+} exchanger is an antiporter located in the plasma membrane that was found to expel Ca^{2+} from the cytosol. Overexpression of the protein might lead back to an increase in cytosolic Ca^{2+} with overexpression of the antiporter in response to regulating intracellular levels of the ion.

Hence, nsPEF treatments could act as a nonchemical trigger for intracellular Ca^{2+} release and consequently Ca^{2+}-dependent signaling pathways. A cytosolic Ca^{2+} increase through release from intracellular storage compartments or through Ca^{2+} influx impacts cell metabolism and growth by transmitting Ca^{2+}-related signals. Cytosolic Ca^{2+} interacts with a myriad of intracellular messengers and signaling proteins through receptors that are responsive to cytosolic Ca^{2+}. Ca^{2+} is involved in a highly complex signaling system, including that associated with phosphorylation processes, the regulation of transcription factors, or the regulation of promoter elements in plants (Kudla et al., 2010). For example, proteins with Ca^{2+}-binding domains located at the plasma membrane, vacuole membrane, nucleus, and cytoplasm can act as Ca^{2+} sensors. Alterations in cytosolic Ca^{2+} concentration can occur depending on the magnitude of the treatment stimulus. Additional temporal and spatial information then creates a rather specific signal signature relevant for further signal processing (Batistič et al., 2010). Su et al. (2015) showed that in plant cells (*H. ammodendron*), nsPEF treatments caused a release of Ca^{2+} from intracellular storage compartments. This release was related to Ca^{2+}-induced upregulation of intracellular signaling pathways and subsequent growth promotion. The authors hypothesized that nsPEF treatment (100 ns, 10 kV cm^{-1}, and 20 kV cm^{-1}, 20 pulses) induced the release of Ca^{2+} from intracellular storage compartments, resulting in the activation of nitric oxide (NO) synthase. NO mediates physiological, developmental, and biochemical processes in plants (Asgher et al., 2017).

Haberkorn et al. (2019) showed that treating axenic *C. vulgaris* at 100 ns, 10 kV cm^{-1}, and 5 Hz significantly increased its biomass yield by up to 17.5 ± 10.5%. The authors hypothesized, that nsPEF treatments could induce a nonchemical, physical triggering of nitrate (NO_3^-)-dependent growth pathways in *C. vulgaris* through the release of Ca^{2+} from intracellular storage compartments. In plant cells, NO_3^- induces Ca^{2+} signaling as a nutrient response mechanism and was shown to regulate plant growth and development, as it is involved in protein and energy metabolism (Liu et al., 2017; Zhao et al., 2018).

5.3. Metabolism and genes

NsPEF treatments were suggested to trigger metabolic pathways as a downstream response to elevated ROS or Ca^{2+} levels, as described in Sections 5.1 and 5.2. Another major metabolic pathway involved in nsPEF related growth stimulation was associated with that of the phosphatidyl inositol (PI) signaling pathway (Fig. 4). PIs are a group of phospholipids found in cell membranes that play a pivotal role in cell division, such as mitotic cell rounding and cell elongation, spindle orientation, cytokinesis, and post-cytokinesis events (Cauvin and Echard, 2015). Hydrolysis of PIs leads to the generation of second messengers, which can act as ligands for proteins, enabling the regulation of ion channel activity, ATPase activity, cytoskeletal dynamics, hormonal and stress signaling (Heilmann, 2009).

Activation of the "classical" PI/PIP/DAG/IP_3/PKC cascade was

reported when treating CHO cells with a single pulse of 600 ns and 16.2 kV cm^{-1}. Tolstykh et al. (2017) and Tolstykh et al. (2013) showed that subjecting CHO cells to nsPEF treatments resulted in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol trisphosphate (IP_3). The accumulation of IP_3 in the cytosol and the subsequent triggering of Ca^{2+} release from internal storage (e.g., from the ER) coupled with the increase in DAG on the inner surface of the plasma membrane led to the activation of protein kinase-c (PKC) (Fig. 4). Triggering of the intracellular Ca^{2+} signal transduction mechanism coupled to the ER via activation of the IP_3 receptor located in the membrane of the ER, leading to a cytoplasmic Ca^{2+} increase and subsequent activation of PKC, is supported by several studies (Beebe et al., 2003; Wheeler and Brownlee, 2008). In contrast, Beebe (2015) showed that nsPEF treatments of Jurkat cells (one/ten pulses, 60 ns at 60 kV cm^{-1}; one/ten pulses at 300 ns and 26 kV cm^{-1}) could also result in an inhibitory effect on the PKC-subunit, where, to initiate proliferation, an activation effect would be needed. The protein kinase superfamily is present in all eukaryotic cells, and protein kinases are involved in myriad cellular functions, including those associated with cell growth and development. Among the protein kinases, MAPKs trigger the cell to initiate cell division (Xu and Zhang, 2015). Ning et al. (2019) related an improvement in the differentiation potential of mesenchymal stem cells (MSC) to the release of Ca^{2+} from intracellular storage compartments through nanopore formation of organelle membranes, which in turn activated the c-Jun N-terminal kinase (JNK) and cAMP response element-binding protein (CREB) signaling pathways (Fig. 4). The JNK signaling pathway in turn is a major signaling pathway belonging to the MAPK signaling pathway, which mediates cellular responses, including gene expression, cytoskeletal protein dynamics, apoptosis, and physiological processes, in eukaryotic cells (Zeke et al., 2016). Although PI hydrolysis and PKC or MAPK signaling also occur in plant cells, no study has substantiated nsPEF-induced PI pathway activation in plants or microalgae. Hence, it remains questionable whether growth-stimulating effects related to PI hydrolysis or depletion could also be induced at a shorter pulse duration in plants or microalgae. Since it was demonstrated that protein kinases are affected by nsPEF, the question arises whether activation can be provoked to induce cell proliferation processes or, if not, where the threshold lies for an inhibitory effect that must be avoided when targeting growth stimulation.

Furthermore, several studies observed a genetic response after nsPEF in different model organisms, such as bacteria (*Streptomyces avermitilis*), cancer cells (HeLa S3 and Jurkat cells), stem cells (MSC), viruses (HeLa SV40), and differentiated tissue cells (normal fibroblast cells and chondrocytes) (Guo et al., 2016; Morotomi-Yano et al., 2012; Ning et al., 2019; Xu and Zhang, 2015). Although genetic effects underlying nsPEF processing remain to be elucidated in detail according to those studies, the different activated gene responses were not of specific relevance regarding increased cell proliferation.

5.4. Cytoskeleton

In eukaryotes, the cytoskeleton constitutes microtubules, microfilaments (polymers of the protein actin), and intermediate filaments. Although all three components hold pivotal roles and function mechanically as stabilizing elements that are involved in the motility system required for intracellular material transport, a majority of studies related to nsPEF processing report on actin-affiliated treatment responses. The actin meshwork is closely associated with the plasma membrane, contributing to its mechanical and electrical properties by the underlying meshwork organization. Cross-linking of actin filaments in the cell cortex reinforces the plasma membrane, generates resistance to cellular deformation, restricts diffusion of cell organelles, and transmits forces (Jékely, 2014).

Influenced by the level of the underlying actin meshwork-based stabilization of the plasma membrane, nsPEF treatments were shown to cause reversible or irreversible electroporation. Berghöfer et al. (2009) showed that nsPEF treatments (1 pulse, 33 kV cm^{-1}) affected the cortical actin meshwork of tobacco BY-2 cells by inducing its dissolution. The drug phalloidin stabilized the actin strands connected to the plasma membrane against dissociation, thereby suppressing irreversible membrane perforation. Subjecting transgenic tobacco BY-2 cells that were modified to contain different levels of actinbundling to nsPEF treatments showed that with greater actin bundling, electroporation of the plasma membrane was reduced (Hohenberger et al., 2011). Frey et al. (2011) hypothesized that nsPEF treatments provoke actin reorganization and consequently lead to increased cell growth. The authors described cytoskeletal actin filament dissolution following nsPEF treatments in tobacco BY-2 cells that consisted of applying a single pulse of 10 ns, 30 kV cm^{-1}, while an increase in the cell mitosis index was observed when applying 25 ns, 10 kV cm^{-1} pulses to the same cells. Hence, nsPEF treatment may indirectly impact membrane permeability via the underlying cytoskeleton, which acts as a mediator.

Actin filaments not only aid in stabilizing the plasma membrane but also have a substantial role during mitosis and cell proliferation. Encroaching in actin polymerization and depolymerization processes, nsPEF treatments might manipulate cellular growth processes and thereby influence the cell division cycle by affecting the actin meshwork structure. Actin filaments support the nucleus to move (and tether) to a defined position before mitosis, contribute to mitotic spindle orientation, or act as markers for the division site of plant cells during cytokinesis (Heng and Koh, 2010; Jékely, 2014). Cell division is coupled with chromosome segregation during eukaryotic cell mitosis, requiring simultaneous contraction of the cellular actomyosin network and relaxation at opposite cell poles to induce cytokinesis, which results in daughter cells with a complete copy of the parental cell genome (Lancaster and Baum, 2014). Stimulating actin reorganization through nsPEF treatments could be an important parameter contributing to increased cell proliferation in eukaryotic single-cells. During normal cell metabolism in eukaryotic cells, the cytoskeleton is subject to constant restructuring and growth. Actin reconstruction is governed through protein-mediated depolymerization of actin filaments, which is responsible for sustaining actin filament turnover (Ono, 2007). Additionally, in plant cells, depolymerization and polymerization of actin filaments contribute to the intracellular motility system. This allows intracellular movement of components, such as organelles and chromosomes (Pollard and Borisy, 2003). Enhanced depolymerization can foster actin turnover and lead to stimulated metabolic activity and eventually mitosis. In human cancer cell lines (Jurkat and HeLa), Stacey et al. (2011) showed that nsPEF treatments (single pulse, 60 ns, 15 kV cm^{-1}) led to electrochemical depolymerization of actin filaments. Electrochemically induced depolymerization of actin filaments was reported to result in delayed cell cycle progression in eukaryotic cells (Stacey et al., 2011). Berghöfer et al. (2009) showed that nsPEF treatments (10 ns, 33 kV cm^{-1}) resulted in the detachment of actin bundles responsible for moving and tethering the nucleus. Consequently, nsPEF treatments triggered an apoptotic response in the treated cells by influencing the onset and outcome of mitosis. In contrast, Kühn et al. (2013) proposed that the actin response following nsPEF treatments (10 pulses, 100 ns, 10 kV cm^{-1}) in tobacco BY-2 cells induced delayed premitotic nuclear positioning followed by temporary mitotic arrest. However, these opposite findings might be related to the different processing parameters applied, resulting in a different energy input for the treatment, as well as other differences in the experimental setup. The observations described for plant cells could be translatable to other eukaryotic single-cells, such as microalgae, owing to the presence of actin in all eukaryotic cells.

Although other structural components might also contribute to nsPEF-enhanced cell proliferation in prokaryotic cells, the involvement of actin homologs was proposed in rod-like prokaryotes, including the bacterial protein MreB. The interaction of the actin homolog MreB with the elongation factor Tu suggests the involvement of the elongation factor as a stabilizing element for prokaryotic cells (Mayer, 2003).

Buchmann et al. (2019) reported the overexpression of the elongation factor Tu following growth-stimulating nsPEF treatments (100 ns, 10 kV cm^{-1}, 9 Hz) of the cyanobacterium *A. platensis*. Analogous to the effects observed in plant cells, the overexpression of the elongation factor could have contributed to reinforcing plasma membrane stability. Accordingly, Buchmann et al. (2019) reported effects on the plasma membrane following nsPEF treatments to be limited.

6. Current applications and future trends of nsPEF in single-cell biorefineries

6.1. Microalgae biorefineries

Although microalgae have attracted attention as a future resource for the bio-based domain, the economic viability of microalgae feedstock production remains hampered by elevated production costs, facing challenges of low upstream productivity (Caporgno and Mathys, 2018; Enzing et al., 2014). NsPEF treatments offer a technology-driven, resource-efficient approach and can contribute to transforming microalgae biorefineries into economically viable concepts by triggering bioconversion efficiencies. NsPEF treatments were shown to leverage the biomass yields of both prokaryotic and eukaryotic organisms. Specific energy inputs of $3 \times 256 \pm 67.5$ J kg^{-1} significantly leveraged *A. platensis* biomass yield by $13.1 \pm 1.6\%$. Simultaneously, the content of the high-value added component phycocyanin ($+19.2 \pm 5.8\%$) was increased, highlighting the potential of nsPEF to leverage compound production (Buchmann et al., 2019). Treatments also significantly increased biomass yields of axenic phototrophic and heterotrophic *C. vulgaris* by up to $17.5 \pm 10.5\%$ (Buchmann and Mathys, 2019; Haberkorn et al., 2019). Pigment and carbohydrate contents were unaffected, while a decrease in protein content was observed for *C. vulgaris*. For both organism groups, *A. platensis* and *C. vulgaris*, growth promotion was observed by applying pulse widths of 100 ns and an electric field strength of 10 kV cm^{-1}.

Industrial-scale microalgae cultivation in photoautotrophic monocultures is not yet a realistic scenario. Maintaining axenic cultures under sterile cultivation conditions would be neither economically nor practically feasible on a large scale. At present, progress in implementing nsPEF as an emerging technology in the cultivation process is prevented by a lack of knowledge of the underlying mechanisms of microalgal-bacterial interactions. This can result in unspecific triggering of organisms and thus nonreproducible treatment outcomes (Buchmann et al., 2018b; Buchmann and Mathys, 2019; Haberkorn et al., 2020a; Haberkorn et al., 2019). Haberkorn et al. (2020b) investigated the potential of nsPEF as a growth stimulus for *C. vulgaris* in nonaxenic cultures and showed that nsPEF treatments (100 ns, 10 kV cm^{-1}, 7 Hz) could trigger the growth of *C. vulgaris*. Simultaneously, the authors highlighted the potential of nsPEF as a tool for the selective inactivation of prokaryotes in microalgal cultures and showed that the desired treatment outcome depended on the underlying microbial community composition. The potential of nsPEF as a tool for targeted contamination control during microalgae cultivation was also shown by Buchmann et al. (2018b). Hence, nsPEF treatments could act as a physical means for cell type-specific proliferation/differentiation or inactivation and could thereby pose a viable alternative to current approaches employing chemical agents.

In contrast to industrial-scale microalgae cultivation facilities, with total yearly costs of up to €33.4 M according to Ruiz et al. (2016), the costs of nsPEF processing units are low. Expenditures required for nsPEF lab-scale treatment facilities were estimated to amount to 90,000 €. Major equipment incorporated in those costs includes nsPEF generators and individual measuring devices, for example, those used to assess mass flow, conductivity, or temperature. Upscaling to industrial-scale nsPEF treatment setups would alter demand on the consumable side, while major equipment and measuring devices remain the same as those used for lab-scale nsPEF treatment units. Installations and maintenance for nsPEF processing unit incorporation into already existing microalgae cultivation facilities require only limited additional costs (Haberkorn et al., 2019).

6.2. Biofilms

Biofilms can be understood as a clustering of microbial cells associated with a surface fringed in a matrix of extracellular polymeric substances (Donlan, 2002). Schaffner et al. (2017) addressed the generation of functional complex materials based on bacterial assemblages by employing novel processing technologies based on 3D printing. Such novel processing technologies enable a spatial display of specific compositions, geometry, and properties. Therefore, they offer novel biotechnological and biomedical applications allowing the printing of "living materials", e.g., bacteria. The printing of living materials offers promising new applications, such as the degradation of pollutants or the production of specific compounds in defined structures that might be relevant for medical applications. The ability of nsPEF treatments might include selective and targeted stimulation or inactivation of certain microbial populations within homogeneous and heterogeneous assemblages, which offers promising new applications for the technology. Additionally, nsPEFs could be employed for stimulation of the formation of specific compounds within those assemblages that could enhance matrix formation. Triggering the exchange of nutrients between cells employing nsPEF processing could pose another option. The application of nsPEF treatment leads to electropermeabilization of mainly intracellular membranes, but with increasing pulse widths, the outer membrane is also affected, which increases the mass transfer of molecules and ions across the membranes (Buchmann and Mathys, 2019; Miklavčič, 2017). Therefore, an exchange of substrates, such as growth stimulating agents or nutrients, could be facilitated and affect growth dynamics.

6.3. Bio-fabrication and medical applications

Ning et al. (2019) demonstrated that applying nsPEF treatments to MSCs increased their differentiation potential and elevated chondrogenesis. Implanting nsPEF-treated MSCs into rats augmented cartilage regeneration *in vivo*. These effects were reported both for treatments at either 100 ns at 10 kV cm^{-1} or 10 ns at 20 kV cm^{-1}. Vadlamani et al. (2019) described the enhancement of cell proliferation and differentiation for osteoblast cells originating from MSCs, which act as precursor cells for bone formation. These observations open new gateways in tissue engineering, focusing on accelerating the differentiation potential of cells. Additionally, the manufacturing of artificial skins, medical patches, and food products employs tissue grafts based on mammalian cells. NsPEFs could act as a physical means for cell type-specific proliferation/differentiation. NsPEF treatments may be incorporated as a pretreatment stage of precursor cells. These cells could subsequently be added to the desired location to develop the tissue of interest.

6.4. In vitro meat

In the context of finding alternatives to conventional meat production for future food provision, *in vitro* meat production has gained momentum, but it is challenging to find innovative solutions that enable scale-up to promote the economic viability of this novel resource (Sharma et al., 2015). The production of *in vitro* meat could be approached using different concepts, providing interaction points for the application of nsPEF treatments. For process optimization, nsPEF treatments could, for example, be applied at an early cultivation stage. One approach for the application of nsPEF treatments is the self-organizing technique, which relies on the proliferation of donor cells in nutrient medium. Here, applying nsPEF treatments to the whole cell suspension or on preselected cell types as an upstream performance enhancer could accelerate cell proliferation. As nsPEF treatments were shown to increase the biomass yield per substrate ($Y_{X/S}$), this could lead

to a reduction in donor cell supply while maintaining output. Epithelial cells that have undergone nsPEF treatment showed increased respiratory activity, indicating cell stimulation (Steuer et al., 2018). These cells might be considered representative of *in vitro* meat since epithelial cells are able to form layers via cell-to-cell interactions. Studies conducted by Ning et al. (2019) and Vadlamani et al. (2019) have also shown that nsPEF treatments could promote mammalian cell proliferation and differentiation (Section 6.3). To investigate the potential of nsPEF treatments to enhance *in vitro* meat production, *S. cerevisiae* could serve as a potential model organism for animal cells.

7. Conclusion

NsPEF processing is a resource-efficient, low-energy-demand technology for enhancing the bioconversion efficiency of single-cell-based biorefineries and can act as a physical means for cell type-specific proliferation/differentiation and compound stimulation. Although the exact working mechanisms remain elusive, evidence suggests that this enhancement is mainly based on the induction of intracellular, abiotic sublethal stress but can also partially be related to effects caused at the plasma membrane level. Effects were associated with electropermeabilization of membranous structures, intracellular and on plasma-membrane levels. Transient cytosolic Ca^{2+} hubs as common denominators among different organism groups, as well as ROS formation, were suggested to be effects that culminate in the onset of cellular response pathways, e.g., those associated with protein kinases. A concrete assignment of definite working mechanisms and their contribution to enhancing bioconversion efficiency, however, remains challenging. Current applications of nsPEF processing include lab-scale microalgae, bacteria, and yeast biorefineries. However, low operating costs and the ease of incorporating nsPEF treatment facilities into single-cell biorefineries suggests promising applications for high-value compound production, pharmaceutical purposes, and in the medical and bioprocess engineering domain. Limitations to industrial-scale nsPEF implementation were not related to technical feasibility but to a lack of understanding of the underlying treatment mechanisms. More research is required to improve the understanding of the cellular mechanisms underlying nsPEF-based bioconversion efficiency enhancement. Better process understanding and reporting and consequently improved process control could foster the upsurge of industrial-scale nsPEF realization and ultimately perpetuate nsPEF applicability within the bio-based domain.

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The work has not been published previously and is not under consideration for publication elsewhere. All authors agreed to the authorship and submission of the manuscript to Biotechnology Advances for peer review. Upon acceptance, the authors agree that the work will not be published elsewhere.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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