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Reversible electroporation caused by pulsed electric field – opportunities and challenges for the food sector

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Abstract

Background

The application of Pulsed Electric Field (PEF) to food may result in reversible or irreversible electroporation of cell membranes, depending on whether cell homeostasis is restored after resealing. Restoration of homeostasis upon reversible electroporation implies the recovery of the pre-pulse transmembrane potential and the restoration of cell metabolic functions. Enhanced membrane permeability caused by reversible electroporation would allow impregnation of cells with foreign molecules and/or stress-induced metabolic reactions. The impregnation of cells and the induction of stress in cells could open new opportunities for the application of PEF in the food industry.

Scope and Approach

Most of the published literature on the application of PEF in food systems focuses on the irreversible process, mainly targeting cold pasteurization or mass/heat transfer enhancement. This review focuses on the application of reversible electroporation to enhance metabolic production of secondary metabolites, to accelerate seed germination and fermentation, and as pre-treatment prior to freezing and drying. Finally, the challenges for industrial application of this technology are discussed.

Key Findings and Conclusions

The application of reversible electroporation as a pre-treatment prior to unit operations in the food industry has the potential to improve the quality of the final product in terms of structure, nutritional value or increased productivity. However, its industrial application faces several challenges, related to difficulties in process optimization, scale-up and equipment design. Therefore, significant efforts are still required to apply reversible electroporation on an industrial scale in the future.

Key words: seed germination, drying, freezing, fermentation, secondary metabolites.

1. Introduction

The application of pulsed electric fields (PEF) to living cells at a certain critical electrical field strength leads to a transient increase in transmembrane potential. When the trans-membrane potential exceeds a critical threshold, the membrane is permeabilized resulting in structural changes in the plasma membrane in the form of hydrophilic pores, causing a temporary loss of cellular homeostasis which involves the inflow of membrane impermeant molecules into the cell and the outflow of molecules from the cell (Kranjc & Miklavčič, 2017). To determine the critical electric field to be applied it is common practice to perform a series of experiments with increasing electric field strength to find boundaries for a specific system (Neuman et al., 1998; Thamkaew and Gómez Galindo, 2020). However, there are reports where techniques such as microfluidics (García et al., 2016) or electroporation system with concentric electrodes in combination with fluorescent probes (Blumrosen et al., 2016) have been used.

Below a critical energy threshold, cells can recover from the loss of homeostasis after the membrane reseals, recovering the pre-pulse transmembrane potential and, consequently, the cells restore their pre-electroporation metabolic functions and survive. This is referred to as reversible electroporation (Wasson et al., 2020). If the applied electric field is above the critical threshold, the cells cannot recover from the loss of homeostasis after resealing, resulting in cell death. This is referred to as irreversible electroporation (Kranjc & Miklavčič, 2017; Wasson et al., 2020) (Figure 1). The

reversibility of the treatment depends on the parameters applied during electroporation such as electric field strength, pulse shape, pulse width, pulse frequency, number of pulses, number of bursts and duration between bursts. It also depends on the characteristics of the cells such as size, shape and conductivity of the cytoplasm (Kotnik et al., 2019).The most common methods to evaluate electroporation are the assessment of the permeabilization of cell membranes on the tissue surface under a fluorescence microscope using Propidium Iodide (PI) (Dymek et al., 2014) and to check the viability of the tissue using various methods, including fluorescent markers, leakage measurements (conductivity), or wilting tests (Dymek et al., 2014; Demyr et al., 2018; Thamkaew and Gómez Galindo, 2020). These methods are also used to differentiate between reversible and irreversible electroporation.

In heterogeneous cell populations and tissues, the effect of electroporation may also be heterogeneous which is part of the challenge in developing appropriate PEF treatments. When PEF is applied at a low field strength (< 200 V) to a heterogeneous cell population, only a fraction of the cell population may be electroporated (Dymek et al., 2015). The amount of reversibly electroporated cells in the population would increase with increasing intensity of the field, to a point where most of the cells in the population are electroporated. This progression of reversible electroporation of cells in a population has been observed in tissues such as potato and basil (Galindo et al., 2009; Thamkaew & Gómez Galindo, 2020). Reversible electroporation allows the cells to survive but causes severe stress and a number of stress-associated metabolic responses are not yet understood (for a review, see Gómez Galindo (2016)).

It has been reported that when certain PEF conditions were applied to spinach leaves grown under controlled conditions in a greenhouse, some leaves in a batch survived the application of PEF and others did not (Demir et al., 2018), suggesting that there may be a narrow range of sensitivity to the applied PEF conditions in heterogeneous commodities that distinguishes between reversible and irreversible electroporation. Even if the electroporation would be irreversible for any cell, at higher electric fields, cell death may be “short-” or “long-term”. Gabriel & Teissié (1995), working on Chinese hamster ovary cells, described “short-term death” associated with high membrane permeability present more than 15 min after pulsation and “long-term death” associated with partial loss of growth ability of resealed cells.

Most literature on the application of PEF treatments in food systems focuses on irreversible electroporation for various purposes such as pasteurization (Evrendilek & Zhang, 2005; Huang &

Wang, 2009; Ranjha et al., 2021), and enhancing of mass transfer in processes such as extraction (Naliyadhara et al., 2022; Ranjha et al., 2021; Vorobiev et al., 2005) and dehydration (Paraskevopoulou et al., 2022; Matys et al., 2022). Little has been published on the applications of reversible electroporation, which is the focus of this review. The application of reversible electroporation in freezing, drying, fermentation and production of secondary metabolites is critically reviewed and the prospects for industrial applications are discussed. The application of reversible PEF to metabolic consequences has been reviewed elsewhere (Gómez Galindo, 2016), and will not be the focus of this paper. In addition, an update on the state of the art in the application of reversible PEF for secondary production metabolites, reviewed elsewhere (Soliva-Fortuny et al., 2009) is provided, focusing on the increased production of secondary metabolites due to PEF-induced stress rather than the use of PEF as a physical tool to enhance their extractability. The aim of this review is to provide a critical overview of the current knowledge on this technology in order to highlight potential applications and challenges related to its future industrial use.

Figure 2 shows a schematic diagram of the various applications of PEF as a function of pulse duration and electric field strength. The main factor that determines the fate of the cell after electroporation, whether it survives or not, is the electric field strength. Electric field pulses of relatively long duration (microseconds or milliseconds) and relatively low amplitude (100 kilovolts per meter) produce biological effects at the cellular level mainly through the formation of pores in the outer cell membrane. High-field pulses with a width of nanoseconds (nsPEF) provoke a different effect on cells because the pulse duration is shorter than the plasma membrane charging time. Instead, the fast-rising electric pulse facilitates the penetration of the electric field into the cell interior, charging intracellular structures. Some reports refer to this method as “electroperturbation” instead of “electroporation” (Beebe & Schoenbach, 2005), although the formation of nanopores in the outer cell membrane has been reported when nsPEF is applied (Vernier et al., 2006).

2. Applications of reversible electroporation in food

Metabolic stress responses to the application of reversible PEF have been investigated to influence the production of secondary metabolites in plant products (García-Parra et al., 2018; Vallverdú-Queralt, Odriozola-Serrano, et al., 2013) and cell cultures (Gueven & Knorr, 2011; Gürsul et al., 2016) as well as stimulating cell proliferation and growth in processes such as fermentation (al Daccache, Koubaa, Salameh, et al., 2020; Mattar et al., 2014) and seed germination (Ahmed et al., 2020; Dymek et al., 2012). The potential of reversible PEF to improve the quality of frozen and dried

plant raw materials has also been demonstrated (Demir et al., 2018; Kwao et al., 2016; Phoon et al., 2008; Telfser & Gómez Galindo, 2019).

2.1. Production of secondary metabolites

An interesting application of reversible electroporation in plant material is the induction of stress resulting in increased endogenous production of secondary metabolites by the tissue. Secondary metabolites are produced by the plant in response to different types of stress and have mainly a protective function, playing an important role in plant adaptation and survival (Halder et al., 2019; Pagare et al., 2015). In addition to photoprotective, signalling and structural stabilizing properties, they also have antimicrobial and antioxidant capacities. The most investigated secondary metabolites are polyphenols and carotenoids, but also glucosinolates in *Brassicaceae* (Balaša, 2014). **Table 1** contains the main results on the production of secondary metabolites induced by the application of PEF in plant tissues and cell cultures, while **Table 2** lists similarities and differences observed among studies performed with the same food matrix with some further critical discussions arisen from the comparison.

Reversible electroporation has been shown to affect cell metabolism and promote the onset of a physiological response. Although this response is at date not fully understood, there are several studies that have attempted to characterize it. Gómez Galindo et al. (2009) used a metabolomic approach to characterize the physiological response of potato tissue to the PEF-induced stress and compare it to wounding stress. The authors reported that the observed response was unique and occurred in the time frame of hours after treatment. However, they did not detect any accumulation of polyphenols or antioxidants. On the other hand, the onset of the enzyme phenylalanine ammonia-lyase (PAL), which is considered a key enzyme of phenylpropanoid metabolism, and therefore a good marker for polyphenol *de novo* synthesis, was observed in many tissues after exposure to reversible electroporation (Balaša, 2014; Gürsul et al., 2016). Balaša (2014) concluded that PEF promote the *de-novo* synthesis of polyphenols in apples, berries and grape cultivars, as well as in apple and grape cell cultures, following an increase in the PAL activity immediately after the treatment and after 9 h. In addition to *de novo* synthesis, the concomitant increase in polyphenoloxidase (PPO) activity suggests that other biosynthetic pathways are also affected by electroporation, although this was not fully elucidated by the authors. Similarly, an increase of PAL activity was observed by Gürsul et al. (2016) in tomato cell cultures exposed to 1 to 9 pulses at electric field strengths between 600 and 1200 V/cm at 4 and 96 hours after the application of the electric treatment.

Cai et al. (2011) studied the effect of PEF on cell cultures of V*itis vinifera* and indicated that the increased yield of secondary metabolites could be the sum of two phenomena, the stimulation of *de-novo* synthesis due to a physiological response that is not yet fully understood and an increase in intracellular metabolites due to changes in membrane functionality. Indeed, as observed by Sotelo et al. (2018), a higher extractability of total polyphenols in sour cherries was observed 24 h after the application of PEF in the range of 0.3-2.5 kV/cm. Reversible electroporation is known to temporarily impair cell membrane functionality, increasing permeability. The duration of this effect is not yet known, as the time required for pore resealing or complete restoration of cell functionality has not been determined. However, it is possible that an increase in the recovery of functional compounds is due in part to increased extractability. It is therefore difficult to separate the effects of the two phenomena, unless more specific investigations on physiological activation and on the cell membrane functionality are carried out.

Various authors (Vallverdú-Queralt, Odriozola-Serrano, et al., 2013; Vallverdú-Queralt, Oms-Oliu, et al., 2013; Soliva-Fortuny et al., 2017; Ribas-Agustí et al., 2019) investigated the bioaccumulation of secondary metabolites considering specific profiles of polyphenols and carotenoids and observed a high variability among the different classes and compounds. A treatment of 0.4 kV/cm, 5 monopolar pulses of 4 μs on whole unpeeled apples followed by 24 h of storage resulted in an increase in hydroxycinnamic acids and flavan-3-ols, while the content of dihydrochalcones and flavonols was not affected (Ribas-Agustí et al., 2019). In apples exposed to combinations of 0.4-2 kV/cm and 5-35 monopolar pulses with energy in the range of 0.008-1.3 kJ/kg followed by 24 h storage, it was found that flavan-3-ols had the highest rate of accumulation compared to total phenolic content and flavonoids (Soliva-Fortuny et al., 2017). The authors observed that the accumulation of secondary metabolites was higher at lower energy input. Vallverdú-Queralt, Odriozola-Serrano, et al., (2013) and Vallverdú-Queralt, Oms-Oliu, et al., (2013) studied the effects of a moderate intensity pulsed electric field (0.4-2 kV/cm using 5-30 monopolar pulses) on the polyphenols and carotenoid profile of tomato. The results showed the bioaccumulation of both in the 24 h following PEF application; however, considering the individual compounds, results were characterized by a high variability. Marked increased were observed for chlorogenic (+ 152%), caffeic acid-O-glucoside (+ 170%) and caffeic (+ 140%) acids for polyphenols and for α-carotene (+93%), 9- (+94%) and 13-cis-lycopene (+140%). The authors hypothesised possible metabolic pathways such as the enhancement of polyphenols biosynthesis, by stimulating the activity of PAL, and of the biosynthetic pathway of carotenoids including the isomerization of *cis*- to *all- trans* forms.

Guderjan, Töpfl, Angersbach, & Knorr, (2005) investigated the effect of reversible electroporation on selected compounds of interest for some oil crops such as olive, maize and soybean after mild drying. The application of electric fields in the range of 0.6-1-3 kV/cm allowed to increase the oil yield in olives and the recovery of isoflavonoids in soybean oil and phytosterols in maize germ oil after drying and an incubation period of up to 24 h after PEF treatment. In contrast, an application of a stronger field (7.3 kV/cm) resulting in irreversible electroporation showed no such effect, suggesting that the higher yield of isoflavonoids and phytosterols was due to a metabolic response rather than cell breakdown. Experiments with maize showed that irreversible electroporation did not achieve the same yield of secondary metabolites, ruling out improved extraction due to electroporation. However, the authors point to the possibility of enhanced extraction of oil components due to loss of water-soluble compounds during the resting time.

An interesting synergistic effect of PEF with other types of stress elicitors was observed. Saw, Riedel, Cai, Kütük, & Smetanska (2012) found that, when combining a PEF treatment (1.6 kV/cm, 10 monopolar pulses) with ethephon, a plant growth regulator, it was possible to enhance the accumulation of anthocyanins in *V. vinifera* cell cultures during the following 14 days of storage. Similarly, Kastell, Schreiner, Knorr, Ulrichs, & Mewis (2018) observed that a two-fold increase in glucosinolate content in *E. sativa* hairy root cultures was obtained after 24 h of the application of the chemical elicitor jasmonic acid combined with the physical elicitor PEF.

The influence of storage temperature after the application of reversible electroporation was also studied. González-Casado, Martín-Belloso, Elez-Martínez, & Soliva-Fortuny, (2018) observed that the concentration of carotenoids in tomatoes stored up to 5 days at 12 °C after PEF treatment (40-200 kV/m, 5 monopolar exponential-wave pulses 0.1 Hz) was significantly higher than in fruits stored at 4 or 20 °C. For polyphenols, the optimal temperature seems to depend on the specific type, as found by Soliva-Fortuny et al. (2017) on apples. The authors observed that the increase in total phenolics (13%) and flavan-3-ol (92%) content in apples was maximised during 24 h at 22 °C, while for flavonoids it was maximised at 4 °C (58%). Nevertheless, the greatest absolute increase in total antioxidant capacity was reached when storing the fruits at 4 °C for 12 h.

The obtained results indicate that the application of PEF for secondary metabolites production enhancement must be optimized for each specific product and compound of interest in terms of treatment parameters. Considering the available literature, conditions for triggering a stress response

generally require a field strength in the range of 0.2-3 kV/cm. However, it is difficult to define the effects of the other parameters, such as pulse width, frequency, and total energy, because very few papers use different ranges for these parameters.

Secondary metabolites of plants often exhibit antioxidant and other biological properties associated with important health benefits (Ribas-Agustí et al., 2019). As the demand for foods with high functional value is constantly increasing, the stimulation of secondary metabolites biosynthesis may represent an interesting opportunity for the food industry needs to meet this request (García-Parra et al., 2018). On the other side, the use of plant cell cultures may provide potential renewable sources of secondary metabolites that could be used as pharmaceuticals, dietary supplements and food additives. The use of cell cultures offers several advantages, such as independence from geographical location, climatic, seasonal and growth conditions and the production of highly uniform products (Gürsul et al., 2016).

However, as mentioned earlier, the yield of secondary metabolites could be due to both increased biosynthesis and increased extractability. Therefore, other markers of metabolic processes should be analysed in addition to extraction yield. In the available literature, although many authors refer to possible metabolic processes, few of them actually test them (e.g., PAL activity). This represents a serious limitation for this application of reversible PEF. Furthermore, while it is clear that some time must elapse before testing for increased metabolite production in order for metabolic processes to occur, significant variations are observed in the time elapsed after PEF treatment, (from a minimum of 4 h to a maximum of 20 days) and in storage conditions (e.g., from 4°C to 22°C). Moreover, all authors focused the attention on the production of specific metabolites of interest without considering whether the enhanced metabolism could lead to the production of toxic compounds. This aspect is particularly relevant if the final product is intended for human consumption, less if the desired compounds are extracted and purified for other uses; however, we believe that these aspects are important for a better understanding of the metabolic consequences of reversible PEF on plant tissues.

Another critical point related to the secondary metabolites production is that it has been studied only on a small scale. Apart from Balaša (2014) who states that 2 kg of apples were considered for each condition applied, other authors do not specify the exact number of fruits/vegetables used, and to our knowledge, the application has not been validated on a- large scale, nor considering different ripening degree, or different cultivars of the same product, that might have a significant effect on the metabolic response of the tissues. Therefore, although the application seems promising, not enough information is currently available. Since in many cases an increased extraction effect cannot be ruled out, much

research is still needed to elucidate the mechanisms involved in these changes, which are currently associated with induced abiotic stress.

2.2. Seed germination

Seed germination is a very important stage in the life of a plant and involves processes that begin with water uptake by the inactive dry seed and end with the development of the embryonic axis (Rifna et al., 2019). The biochemical and physiological responses that occur during germination are influenced by various factors, both intrinsic and extrinsic. Induction of seed germination can be used to improve crop yield and quality, to ensure global food security and meet the increase demand for highly nutritional foods (Edmondson et al., 2014; Leong et al., 2016).

There are a variety of physical and chemical methods that can be used to increase germination power and growth rate. Recently, Rifna et al. (2019) provided a review of emerging technologies for improving seed germination, describing reversible PEF as one of the possible technologies. Examples of the impact of the application of electric pulses on seed germination are summarized in **Table 3**, while similarities and differences observed among studies conducted on the same food matrix in **Table 4** are listed with some further critical discussions arisen from the comparison.

Reversible PEF was applied as a method for 'electropriming' of wheatgrass seeds with the aim of increasing the bioprotective properties of the resulting shoots (Leong et al., 2016). The results showed that the higher applied electric field strength (2 kV/cm with 20 μs pulse width) stimulated the endogenous antioxidant response of the tissue, resulting in an increased glutathione levels and activity of various enzymes related to antioxidant metabolism in plant cells, with only slight inhibition of growth potential. The bioprotective effect of the shoots was also confirmed also by studies on Caco-2 cells. The authors highlighted the importance of the cell hydration level before PEF application.

A similar approach was taken by Ahmed et al., (2020) who applied PEF to wheat (*Tritium aestivum* L.) seeds at an electric field in the range of 2 to 6 kV/cm, with a number of pulses of 25 and 50. However, in this case, the treatment was applied before the imbibition step. Various indices related to germination (growth, vigor index, water uptake, juice yield) and some nutritional properties were evaluated (content of soluble proteins, chlorophyll, amino acids, minerals, phenolic and antioxidant activity). Seeds were able to absorb water more quickly during imbibition, resulting in faster

germination proportionally to treatment intensity and greater leaf area for the 6 kV/cm treatment. Moreover, PEF allowed to increase the content of antioxidant compounds in the resultant seedlings.

The effects of PEF treatment based on monopolar rectangular pulses with varying frequency, treatment duration and total energy as an alternative to chemical treatments on the surface disinfection, germination rate, and tolerance to cold and salt stress in wheat seeds were assessed (Evrendilek et al., 2021). PEF treatments allowed to significantly increased germination and seedling rates by 10 and 28%, respectively compared to the untreated sample, and improved vigor. Moreover, higher tolerance to cold and salt stress and a reduction in endogenous microflora of different microorganism were observed due to PEF application.

The metabolic response of barley seeds to the application of PEF at different voltages up to 1.2 kV/cm was investigated (Dymek et al., 2012). Growth impairment was observed with a reduction in radical elongation at higher electric field. However, gross metabolism measured by isothermal calorimetry was not significantly affected, indicating that the tissue retained its ability to perform metabolic processes. Interestingly, the study of protein patterns showed a lower accumulation of α-amylase, which may have caused a reduced availability of sugars from starch during germination.

The application of very short pulses, of the ns duration, has also been tested on seed germination. PEF has different effects on cells depending on the pulse duration. PEF with milliseconds to pulse duration of microseconds is commonly used for electroporation, because it acts primarily on the cell membrane to create pores suitable for the transfer of macromolecules. PEF with ns pulse duration (nsPEF) is applied to generate small pores on the cell membrane to allow permeation of small molecules such as ions and water. nsPEF treatment also acts directly on intracellular components. Compared to μs pulses, ns pulses are shorter compared to the charging time of the membrane, and therefore the electric field is conducted through the intracellular space and affects internal cell organelles (Buchmann et al., 2019). Reported effects of nsPEF application include increased number of minimum size pores compared to conventional PEF (Gowrishankar et al., 2006), a faster pore formation (Sridhara & Joshi, 2014) and an increase in the inner mitochondrial membrane permeability (Napotnik et al., 2012) were observed. Examples of nsPEF applications with reversible effect are described below for seed germination. While reversible electroporation with μs pulses shows effects on seed germination through increased content of antioxidant compounds in seedlings, faster water uptake and acceleration of growth, reversible electroporation with nsPEF typically causes a stress

response with stimulatory effects on growth during seed germination (Eing et al., 2009; Songnuan & Kirawanich, 2012; Su et al., 2015).

When 10 ns pulses with an intensity in the range of 5-20 kV/cm were applied to *Arabidopsis* seeds (Songnuan & Kirawanich, 2012), the average leaf area increased up to 50% when 10 kV/cm was applied, while after 2 weeks, a significant effect was observed for all samples compared to the control. However, the 10 kV/cm allowed to maximize the leaf area up to 80%.

The effects of nsPEF treatment, testing several conditions of field strength and total specific energy on phenotypic changes of seven days old *Arabidopsis thaliana* seedlings were studied (Eing et al., 2009). The authors observed a growth-promoting effect that resulted in increased leaf area at a field strength of 5 kV/cm for all pulse durations and pulse numbers. Increasing electric field reduced the stimulatory effect until growth inhibition at 50 kV/cm. The growth simulation was attributed to a stress response of the plant system.

Seedlings of *Haloxylon ammodendron*, a dominant desert plant, were exposed to nsPEF in the range 10-30 kV/cm with 100 ns pulse width to investigate the effects on early physiological development (Su et al., 2015). They observed that the lower field strengths studied played a significant role, by increasing the generation of nitric oxide (NO), an important signalling molecule that exerts various physiological functions in plants, which was associated with stimulated germination and growth. However, an excessive NO production, obtained at the 30 kV/cm treatment resulted in a decrease in germination rate. The reduction in oxidation-reduction potential (ORP) after nsPEF-seeds treatment was also involved in stimulating seed growth. Compared to studies on Arabidopsis, the authors monitored the effect only up to 48 h after PEF.

Considering the published literature, all authors suggest that PEF can improve seed germination and growth rate by stimulating a physiological response involving ROS production and oxidative stress, however, the exact mechanism is still largely unknown. According to the published studies, PEF produce an abiotic stress to tissues that activates multiple metabolic processes, but each author focused on selected processes, while a comprehensive understanding is lacking. Authors frequently mention the increased release of calcium from the endoplasmic reticulum, as observed in animal cells (Su et al., 2015); however, this phenomenon has not been demonstrated in plant tissues.

It is notable that, for plant tissue, the range for reversible electroporation is considered approximately 0.5-1.5 kV/cm (Raso et al., 2016), whereas, it seems to be broader in seeds, up to 6 kV/cm with a pulse width in the range of µs (Ahmed et al., 2020), and up to 50 kV/cm when using pulses width in the range of ns (Eing et al., 2009). However, it is yet difficult to define recommended parameters for seed germination, because very few studies are available.

2.3. Fermentation

Fermentation is used as a food preservation method that involves the chemical conversion of complex organic compounds into simpler compounds through the growth and metabolic activity of microorganisms and the activity of microbial enzymes (Di Cagno et al., 2013). For the food industry, it is important to stabilize microbial growth and increase the process productivity as well as the fermentation yield (Ribéreau-Gayon et al., 2006). Recently, the fermentation process has been used for the production and extraction of specific bioactive metabolites for the food, pharmaceutical and chemical industries. Among the secondary metabolites produced during fermentation, there are various compounds with known bioactivity such as peptides, sugars, and antibiotics.

For the production of specific compounds of interest (e.g., secondary metabolites selected for their economic or nutritional value), the control and enhancement of the fermentation process is critical. To influence the behavior and growth kinetics of microorganisms, non-conventional methods such as PEF technology are increasingly being tested. **Table 5** shows examples of the influence of the application of electric pulses on fermentation. For studies conducted on the same class of microorganisms, the similarities and differences are listed in **Table 6** arisen from the comparison.

The typical application of PEF technology for cold pasteurization of food products involves the application of high voltages (20-80 kV/cm), that cause irreversible destruction of the microorganisms. On the other hand, milder conditions can be applied to affect microbial growth and fermentation. Reversible electroporation of the cell membrane increases the diffusion of ions and molecules through the pores in the cell membrane, which enhances nutrient uptake (Mota et al., 2018). This uptake promotes the cellular growth and fermentation process. Some authors, using transcriptomics (Tanino et al., 2012), and proteomic analysis (Buchmann et al., 2019) identified possible stress response pathways activated by reversible electroporation.

A more efficient fermentation process was observed in terms of cellobiose utilization by *Kluyveromyces marxianus IMB3*, a thermotolerant yeast strain, when pulses of 0.25kV for 10 ms were applied. After application of the pulses, the saline buffer and the cells were added to the yeast

growth medium and incubated to measure ethanol production (McCabe et al., 1995). The conversion of the cellobiose substrate to ethanol increased by 40%. (Fologea, Vassu-Dimov, Stoica, Csutak, & Radu, 1998) investigated the survival of *Saccharomyces cerevisiae* after electroporation with bipolar pulses in the range of 0 - 1.5 kV/cm electric field strength. After PEF treatment, cells were inoculated and incubated in YEPG (Yeast Extract Peptone Glycerol) medium and then plated out to record the growth rate. The growth rate was accelerated by electroporation and peaked at a field strength of 0.85 kV/cm.

Most studies published on the application of PEF in microbial fermentation focused on *S. cerevisiae* as the target microorganism, which is probably the most commonly used yeast. Other investigated yeasts include *Hanseniaspora* sp. (Al Daccache, Koubaa, Maroun, et al., 2020; Al Daccache, Koubaa, Salameh, et al., 2020), and *Aspergillus niger* (Fiedurek, 1999).

The effects of different electric field applications between 0.1 – 6.0 kV/cm, 1000 pulses with 100 µs pulse duration, and 100 ms pulse repetition time were investigated on wine yeasts (*Saccharomyces cerevisiae*). After the electric treatment, the cell suspension was agitated and inoculated into the fermentation substrate. The results clearly showed a positive impact of PEF treatment resulting in an enhancement of the fermentation kinetics. Electrostimulation was confirmed by the increase in electrical conductivity of the yeast suspensions after each treatment. Reversible electroporation of yeast cells accelerated sugar consumption in the initial stage of fermentation (in the lag phase). Consumption of fructose was nearly 2.33 times higher compared to control at 0.1 kV/cm and 3.98 times at 6.0 kV/cm. The application of PEF also accelerated biomass growth and increased protein synthesis during the fermentation process. At the end of the fermentation (at the beginning of the declination phase), due to the decrease of essential nutrients and the formation of inhibitory products (e.g., organic acids), there was a limitation of the fermentation that resulted in a 30% mass reduction for the samples treated with 6.0 kV/cm, while 20 additional fermentation hours were required for the same reduction for the control samples. The PEF treated yeast suspension exhibited faster kinetics of fermentation compared to the control yeast suspension (Mattar et al., 2015).

Other important microorganisms that have been studied include various strains of lactic acid bacteria (Ewe et al., 2012; Góral et al., 2019; Kanafusa et al., 2021; Lye et al., 2011; Najim & Aryana, 2013; Vaessen et al., 2018; Yeo et al., 2014; Yeo & Liong, 2013) and, only recently, microalgae (Buchmann et al., 2019; Haberkorn et al., 2019). A common effect observed by many authors after reversible electroporation is the increase in cell growth.

PEF adversely affected cell viability and metabolic processes of various strains of lactobacilli and bifidobacteria immediately after the treatment due to injuries to the cell membrane. After electroporation, the treated cells in this case were transferred to soymilk to follow the growth and bioactivity during fermentation (Yeo et al., 2014; Yeo & Liong, 2013). During fermentation, the cells that survived the PEF treatment (which were only reversibly electroporated) showed higher viability and propagation rates. This effect was attributed to the changes in membrane permeability promoted by electroporation, which allowed a faster diffusion of molecules and ions, promoting an efficient nutrient transport, which in turn led to faster propagation and reproduction during fermentation. Seratlić et al. (2013) confirmed that the population of *L. plantarum* that survived PEF treatment was characterized by a faster growth rate and resistance to further PEF applications, suggesting defence-related consequences of PEF-induced sub-lethal stress.

The effect of PEF treatment on yogurt starter cultures (a mixture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. Bulgaricus*) was investigated in terms of the acidification capacity in reconstituted skim milk medium using an experimental design that included field strength, frequency and pulse number. It was found that the latter was the most influencing variable. Starter cultures were treated with PEF and inoculated into skim milk fermentation bottles. The application of PEF allowed an earlier onset of pH reduction in the fermentation which from an industrial point of view, is a desirable characteristic (Chanos et al., 2020).

Increased cell viability and proliferation rate was also observed in *Streptomyces avermitilis* treated with 10 kV/cm and 20 pulses to enhance avermectin fermentation (Guo et al., 2016) and increased growth in Lactobacilli treated with 7.5 kV/cm for 4 ms, which significantly increased the removal of cholesterol upon treatment and after fermentation from the medium for strains *L. acidophilus* FTCC 0291, *Lactobacillus bulgaricus* FTCC 0411, and *Lactobacillus casei* BT 1268 (Lye et al., 2011). Similarly, aiming at increasing the performance of microalgae-based biorefineries, higher biomass yield of *Chlorella vulgaris* (10 kV/cm, pulses of 100 ns, 5 Hz) and *Arthrospira platensis* (pulses of 100 ns, energy input of 256 kJ/kg) was obtained using nsPEF by Habekorn et al. (2019) and Buchmann et al. (2019), respectively.

Besides growth stimulation, one of the most interesting effects of reversible electroporation on microorganisms is the modification of their metabolism, which may lead to the production of specific metabolites of interest. PEF treatment was applied to spores of *A. niger* with an electric field in the range of 0.57-2.85 kV/cm to assess the production of citric acid, a compound widely used in the

pharmaceutical, cosmetic, food and beverage industries and usually obtained by an industrial-scale process of fermentation. After treatment, the cells were used as inoculum for citric acid fermentation. With the optimal treatment parameters (2.85 kV/cm, 1 ms and 1 Hz), the production of citric acid was increased by up to 1.4 times compared to the untreated sample (Fiedurek, 1999).

Some interesting results obtained after application of reversible electroporation were also observed in terms of probiotic functionality of various microbial strains. PEF was applied with 1 kV/cm electric field and monopolar pulses of 3 µs to cultures of *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* ssp. b*ulgaricus* in a buffer solution (prepared from of 10 ml from a stock solution culture of *Lb. delbrueckii ssp. bulgaricus LB-12* and *Lb. acidophilus LA-K* in 990 ml of sterile 0.1% peptone water). The samples (control and PEF treated) were inoculated in sterilized skim milk to measure the growth, acid tolerance and protease activity. The results showed increased exponential growth, improved acid tolerance and higher proteolytic activity for both strains, which are among the most commonly used strains for fermented milk production, suggesting that PEF treatment can be used to improve the beneficial properties of probiotic strains (Najim & Aryana, 2013).

A nsPEF treatment was applied to *L. plantarum* before fermentation of watermelon juice to obtain a probiotic drink. nsPEF was applied during the log growth phase by modulating the parameters of electric field in the range of 4-6 kV/cm, the number of pulses between 100 and 600 with 35 ns pulse width and the frequency in the range of 1-50 Hz. After treatment, the watermelon juice was incubated to observe cell viability, study cell growth and analyse lactic acid and acetic acid. Depending on the applied voltage, the authors observed a stimulatory effect on the microbial metabolism, with increased production of L-lactic acid, D-lactic acid and acetic acid corresponding to the parameters of 5.0 kV/700 pulses, 4.5 kV/700 pulses and 4.5 kV/1000 pulses, respectively. This increase on metabolites production occurred without affecting cell viability and cell count (Kanafusa et al., 2021).

The production of exopolysaccharides (EPS), components that affects the rheology of fermented milk but also have various positive health effects on humans, was investigated during fermentation by L. *lactis subsp. Cremoris*. PEF treatment with 8 kV/cm, 200 pulses with a pulse-length of 1 µs was performed as single treatment and a circular treatment for 4 h, resulting in a 32% and 94% increase in EPS yield, respectively. The PEF treated samples were cooled to fermentation temperature to continue the fermentation. The molecular size of EPS produced after PEF treatment was smaller, indicating disruption of EPS biosynthesis pathways (Ohba et al., 2016).

Additionally, the impact of PEF treatment (2.5-7.5 kV/cm, 3-4.5 ms) was studied on the ability of some lactobacili strains (*Lb. acidophilus* BT1088, *Lb. acidophilus* FTCC 0291, *Lb. bulgaricus* FTCC 0411, *Lb. bulgaricus* FTDC 1311, Lb. casei BT 1268) to remove cholesterol by incorporation into their cell membrane. The cultures were used for fermentation. The results showed that increasing membrane permeability facilitated the incorporation of cholesterol from the fermentation medium into the cytoplasm, thus enhancing the health benefits (cholesterol reduction) associated with the microorganism (Lye et al., 2011).

Mineral accumulation during fermentation has also been studied. Góral et al. (2019) applied PEF treatment to *Lactobacillus rhamnosus B 442* to maximise the accumulation of zinc. At a field strength of 3.0 kV/cm with a pulse width of 20 μs, and an electroporation time of 15 min, a concentration of 500 μg/mL was achieved in the medium after 20 h of culturing. Similarly, it was observed that exposure to 3.0 kV/cm, 10 μs pulses, and 1 Hz for 10 min were the optimal conditions for the maximising selenium and zinc accumulation in *S. cerevisiae* (Pankiewicz et al., 2017). The authors suggested the use of the yeast as such as a dietary supplement or for the possible production of a functional bread. However, to our knowledge, this application has not yet been tested.

Several studies also addressed PEF-enhanced enzymatic activity of β-glucosidase in lactic acid bacteria (various *Lactobacillus* and *Bidobacterium* strains). PEF treatment with voltages in the range 2.5-7.5 kV/cm and pulse duration in the range 3-4.5 ms prior to inoculation and fermentation in biotin-supplemented soymilk and mannitol-soy milk allowed an improved conversion of isoflavone glucosides into the bioactive aglycones (Ewe et al., 2012; Yeo et al., 2014; Yeo & Liong, 2013). Also, various probiotic properties, such as tolerance to acidic and intestinal bile salt conditions and antimicrobial activity against pathogens were improved, confirming the potential of PEF treatment for the development of functional probiotic products. Compounds of interest include avermectins and their analogues, which are fermentation products of a gram-positive bacterium named *Streptomyces avermitilis*. Avermectins can be used in agriculture, animal health, and human infection control, and are therefore important commercial antiparasitic agents. Application of nsPEF in the range of 5 to 30 kV/cm, 20 to 100 pulses with a width of 100ns, to *S. avermitilis* cultures significantly improved avermectin production. This result was attributed to an enhancement of the microbial cell growth but also to a regulation of the avermectin biosynthesis, through effects on gene expression (Guo et al., 2016).

Finally, the effect of applying electric pulses at different stages of fermentation was investigated. PEF treatment was performed with voltages in the range 72–285 V/cm, and with trains of 10 pulses of 100 µs, applied to *Hanseniaspora sp*. before or during the fermentation of apple juice. The authors observed an increase in biomass growth and a significant reduction in ethanol yield in all the treatments, which is an interesting aspect for the production of fermented functional beverages. Interestingly, they found that the yeast cells were more sensitive to PEF treatment during the lag and early exponential phase of fermentation rather than during the log phase, although the same energy consumption of PEF treatment was considered (Al Daccache, Koubaa, Maroun, et al., 2020; Al Daccache, Koubaa, Salameh, et al., 2020).

The effect of applying nsPEF on different cell growth stages of the microalga *Arthrospira platensis* was evaluated. Cell proliferation was assessed 12, 36 and 60 h after inoculation and the results showed that the increasing effect of biomass growth corresponded to the exponential phase (36 h), After treatment, the microalgae was proposed for use in photoautotrophic microalgae biorefineries (Buchmann et al., 2019). These results demonstrate the importance of optimizing the PEF treatment considering the optimal stage of fermentation.

In summary, extensive literature results show that reversible PEF can stimulate fermentation processes to increase growth rate and metabolites production. It has been suggested that the modifications in cytoplasmic membranes due to external electric fields strength may result in increased transport of beneficial nutrients from the growth medium across the membrane due to the formation of pores and also to the activation of proteins that act as transporters (Barba et al., 2015). This could explain the increased growth and fermentation rate, but the mechanism for the increased production of some specific metabolites needs further study. However, the specific PEF parameters to be used depend on the type of microorganism investigated and, therefore, careful optimization of the process is required. Moreover, even for the most studied microorganisms (S. cerevisiae and Lactobacilli spp.) a variety of operating conditions have been tested (e.g., different voltage and pulse width ranges), thus making it difficult to specify optimal ranges even for one microbial species. Another aspect to note is that all authors applied PEF just before or just after inoculation and evaluated the effects during the first stage of fermentation, rather than throughout the whole process. Finally, the studies only consider the effects on inoculated microorganisms on previously sterilized medium, but an enhancement of metabolism probably also takes place in natural/wild microorganisms, although this has not been specifically investigated.

2.4. Improvement of Freezing tolerance

When fruits and vegetables are frozen, freezing damage to tissue occurs due to ice crystals formation. The direct effect of the formed ice crystals is the mechanical action that leads to rupture of cell membranes. The indirect effects are the migration of water from the intercellular spaces to the extracellular spaces resulting in cell dehydration, shrinkage, and membrane damage. As a result, cell disruption, texture and quality loss (colour, flavour, texture, and nutrients) occur in fruit and vegetables (Alabi et al., 2022).

Efforts have been made to develop pre-treatment methods prior to freezing to improve the quality of products that currently cannot be frozen without drastic quality loss, and where material losses in the food chain are high. Blanching is commonly used prior to freezing especially for vegetables, to inactivate enzymes and prevent enzymatic browning. Blanching affects the texture quality of products (van der Sman, 2020); it causes tissue damage and a textural change, loss of nutrients in the blanching medium and colour change (Jha et al., 2019). Dehydration can be performed prior to freezing to improve the quality of frozen fruits and vegetables. Dehydration can be applied either by conventional air drying or by osmotic dehydration (OD). To improve the quality of frozen fruits and vegetables, the amount of water to be removed by dehydration should be in the range of 30-50% (James et al., 2014), and it should be performed in a controlled manner at low temperature. OD is performed by dipping the products in a solution containing high concentration of solutes (van der Sman, 2020). However, OD damages cells by loss of cell turgor (Giannakourou et al., 2020). Dehydration is a slow process (van der Sman, 2020) and OD can lead to the loss of nutrients such as proteins, minerals, and vitamins (Dziki, 2020).

One of the improvements in the processing steps of frozen fruits and vegetables is the enhancement of OD by vacuum infusion (VI). During VI, the air in the intercellular space of the plant tissue is replaced by an osmotic solution (Şahin & Öztürk, 2016). The osmotic solution may contain other ingredients such as $CaCl_2$ and/or PME to enhance the mechanical strength of the cell wall (Shayanfar et al., 2014; Vaessen et al., 2018). Another improvement is to apply the combination of dehydration techniques with PEF with the purpose of increasing the permeability of the cell membrane of fruits and vegetables (Paraskevopoulou et al., 2022). The application of mild PEF treatment can increase cell membrane permeability, resulting in a facilitated uptake of cryoprotectants, and thus to a better preservation of the texture of the product (Shayanfar et al., 2014). However, the duration of increased permeability is often not determined experimentally.

Investigations on the use of reversible PEF in freezing are scarce in the literature and only few data are available. The aim of using reversible instead of irreversible PEF is to keep the cells intact and keep viability after the application of PEF (Gómez Galindo, 2008). The application of reversible electroporation in combination with vacuum infusion could serve to maintain cell viability after the pre-treatments as well as after a freeze/thaw cycle. Phoon, Gómez Galindo, Vicente, & Dejmek, (2008) showed that PEF in combination with vacuum infusion improved the freezing tolerance of spinach leaves. Vacuum infusion was used to introduce trehalose into the extracellular space of spinach leaves and PEF was used to accelerate mass transfer into the intercellular space by affecting plasma membrane permeability. This experiment was successful in improving the freezing tolerance and preserving the cell viability in spinach tissue even after a freeze/thaw cycle. The application of these combined technologies prior to freezing was patented by the same group in 2013 (Dejmek, Sjöholm, Gómez Galindo, & Phoon, 2013).

Velickova et al. (2018) studied the same combination of unit operations (vacuum infusion and PEF) on strawberries, adding an anti-freeze protein to the trehalose solution. Reversible electroporation of strawberry cells in combination with vacuum infusion improved the viability of the surface cells (epidermal cells) after the freeze/thaw cycle compared to the untreated control and vacuum infusion treatment alone. Survival of cells was evaluated by microscopic observation. Reversible electroporation also improved the retention of internal flesh colour (vivid red) compared to the control after freezing and thawing. However, the PEF conditions applied did not improve the texture or drip loss after thawing compared to strawberries that were only vacuum infused or to the control. This is the only study in the literature on strawberries, no investigation on the effects of other parameters affecting freezing survival has been carried out, as in the case with spinach.

One of the major challenges with these studies is the complex heterogeneous structure of plant tissues, which may not allow for uniform reversible electroporation through the plant cross-section. Dymek et al. (2015) addressed this problem by mathematically modelling the electroporation of the complex heterogeneous structure of spinach leaves. In this model, it was shown that the properties of the tissue, e.g., its conductivity, affect the degree of electroporation of the tissue after reversible PEF application. The surface cells of spinach start to be electroporated at low voltages (50 V) and uniform electroporation of the cells in the other tissues occurs at higher voltages (300 -500 V/cm).

Apart from the parameters applied in the PEF treatment and vacuum infusion, there are several stress factors that affect tissue survival during freezing and thawing. The cultivation temperature of baby spinach leaves was altered from 20 to 5 °C, to test whether inducing cold stress before harvesting and processing would improve survival after freezing and thawing. The combined application of vacuum infusion and PEF was applied as a pre-treatment prior to freezing. Leaf survival increased from 55 ± 5% to 85 ± 4% when cold stress was induced prior to harvest. An "all-or-nothing" effect was observed when assessing leaf survival after freezing and thawing. Either the leaves survived, or they did not (Demir et al., 2018). The application of reversible electroporation to the cold stressed and impregnated leaves may have induced additional stress, resulting in the increase in freezing tolerance of the leaves. All these multiple stress factors may alter leaf ultrastructure, enzyme activities, membrane lipid composition and ion channel activities. Thus, cells may gain protection against freezing stress via co-expression of stress responses.

Table 7 lists the reversible PEF parameters applied to the plant materials before freezing with the main results. As the different studies were conducted on spinach, the similarities and differences observed among them are listed in **Table 8** with some further critical discussions arisen from the comparison.

Considering the available literature, the application of reversible electroporation before freezing together with other unit operations seems to improve freezing tolerance. However, 100% survival has never been achieved after a freeze/thaw cycle**.** Also, as noted by the same authors (Demir et al., 2018), the application of cold stress prior to freezing appears to be necessary to achieve a significant improvement in freezing tolerance; on the other hand, it is unrealistic to assume that the industry could change the growing temperature of the plants in sufficient quantity to meet production volumes of frozen leaves. The authors attempted using different sugars as osmotically active substances (such as trehalose, sucrose, glucose and fructose) in the impregnation solution to increase freezing tolerance, but this strategy did not seem to improve the result compared with the combination of cold stress application followed by vacuum impregnation with different sugars and reversible electroporation. Therefore, to date, there is no viable solution for the industrial application of PEF to improve freezing tolerance. In addition, the authors limited themselves to assessing the viability immediately after thawing and did not consider what happens during subsequent storage. Considering the importance of improving food freezing tolerance for the food industry, further efforts should be made to increase the survival rate by combining different stress factors using a process suitable for industrial needs.

2.5. Drying through stomatal opening

Drying is one of the oldest and most efficient methods of preserving food products. Drying reduces the moisture content of the product to a level where microbial growth is not possible (Iheonye et al., 2023). There are different drying methods, including air drying, vacuum drying and freeze drying. Each drying method has its drawbacks and challenges. Air drying requires long drying times. resulting in degradation of product quality, including colour, odour, flavour and nutritional value. Vacuum drying allows to lower the drying temperature, hence preserving better quality compared to conventional air-drying. However, the low pressure also reduces the heat transfer rate thus increasing drying time. For this reason, vacuum drying is often coupled with a complementary step such as freeze drying. Freeze drying of food products results in better-quality attributes including better colour retention and better rehydration properties. However, vacuum drying and freeze drying also require long drying times and high energy consumption (Huang & Zhang, 2012). Prior to drying, various processes, including PEF, have been applied to improve the quality attributes of dried products, by reducing the time and temperature of the process, thereby reducing the degradation of bioactive compounds, and improving sensory properties. PEF has been used as an irreversible electroporation treatment for a number of food raw materials prior to drying (Iaccheri et al., 2021; Nowacka et al., 2019; Wiktor et al., 2016). However, reversible electroporation prior to drying has not been investigated as extensively because the effect of reversible electroporation on different cell types in plant tissues is a relatively recent finding (Kwao et al., 2016). **Table 9** reports the reversible PEF parameters applied to plant materials prior to drying with the main results, similarities and differences observed among them and further comments are given in **Table 10.**

Plant leaves have stomata on the surface, that control water and gas exchange between the cells and the external environment. Stomatal guard cells are paired epidermal cells shaped like a kidney and surround the stomatal pore (Glover et al., 2016), which can be permanently opened by a sufficient pulsed electric field, promoting water loss from the tissue (Thamkaew & Gómez Galindo, 2020). Stomata opening was studied by applying of PEF with different parameters to basil leaves prior to air-drying at 50 °C. PEF parameters were selected to obtain reversible electroporation of the tissue (600 V/cm), with or without stomata opening (varying pulse duration and spacing) and to obtain irreversible electroporation (1.5 kV/cm) leading to cell death (Kwao et al., 2016). The effects of the different PEF protocols were evaluated by measuring the drying times, concentration of aroma compounds on the dried products, colour and rehydration properties. The results showed that

reversible electroporation, applied to the tissue in such a way as to electroporate the guard cells of the stomata, reduced the drying time by 37% and gave the product to keep better-quality, including aroma compounds, colour and rehydration ability, compared to the controls. Irreversible electroporation provided a faster drying process than all reversible electroporation methods. However, it was reported that the leaves lost more colour and aroma compounds compared to the reversibly electroporated basil leaves. Stomata opening was induced by modulating pulse width and spacing within a narrow range of processing conditions that is close to the limit between reversible and irreversible electroporation (Thamkaew & Gómez Galindo, 2020). The limit between reversible and irreversible electroporation was determined by observing cell survival under a fluorescent microscope using fluorescein diacetate as vital staining. Drying of basil leaves after reversible electroporation is highly facilitated by the opened stomata, whereas irreversible electroporation of plant tissues leads to disruption of cells resulting in mass transfer through the tissue. Reversible electroporation allows for a more controlled drying process with less cell damage to the tissue. Less cell damage leads to a higher rehydration capacity and less leakage into the intercellular spaces, which in turn leads to less enzymatic degradation of aroma compounds and higher retention of colour compounds (Kwao et al., 2016; Thamkaew & Gómez Galindo, 2020).

The effect of reversible electroporation combined with different drying methods, including air drying at 40 °C, vacuum drying and freeze drying on the structure and sensory quality of dried basil leaves was investigated. Reversible electroporation parameters were applied to electroporate the guard cells in the stomata complex (650 V/cm, 65 pulses with 150 µs pulse width, 760 µs between pulses). The results showed that drying times were shortened the most for air dried products (57%), followed by vacuum drying (33%) and freeze-drying (25%) compared to non-electroporated control samples. Dried samples that were reversibly electroporated and vacuum dried were closest to fresh leaves in terms of colour and aroma, according to the sensory panel. Therefore, the effect of PEF also seems to depend on the drying method (Telfser & Gómez Galindo, 2019).

Summarising, the use of PEF provides a shorter drying time. Reversible PEF leads to a better quality of the final product. It can therefore be considered as a useful pre-treatment for high value products, where aroma and colour are particularly important. However, the advantages of reversible electroporation were obtained when the food was dried at low temperatures (40-50°C), which is much lower compared to the typical air-drying temperatures (70-120°C) commonly used in the food industry when the drying process is also aimed at reducing the microbial load of the raw material. The stomatal

opening strategy is limited to leafy vegetables, however, still with potential to a wide range of products (Thamkaew et al., 2020).

2.6. Extraction of compounds

Extraction could be an interesting application area for reversible electroporation. While chemical lysis is highly efficient, it is not suited for food, personal care products and cosmetics, as these sectors need high purity and solvent-free extraction techniques. Moreover, techniques that result in cell lysis and disintegration lead to high quantity of debris released in the extracts that need to be further fractionated and purified (Eleršek et al., 2020). Green extraction is a concept used for energy efficient, solvent-free, non-destructive (when possible) methods/processes for extraction of high-quality compounds (Chemat et al., 2012). Reversible electroporation, as a green extraction technique (solvent-free, energy-efficient, and non-destructive) was investigated for the extraction of proteins and lipids from *Chlorella vulgaris in* comparison to chemical lysis and mechanical disintegration (Eleršek et al., 2020). Algal culture containing the unicellular microalga *Chlorella vulgaris* SAG 211-11b was circulated in an electro flow chamber at 0.72 L/min and electroporated with unipolar square wave pulses at 3-4 kV voltage, 100 µs to 1 ms pulse width, 10 Hz, for 30 min. After the electroporation, the algal cell growth was at the same level as the negative control (no-treatment) after a few days, indicating that the applied parameters were non-destructive. The extraction of protein was lower compared to that obtained after chemical lysis and mechanical disintegration, however simultaneous extraction of proteins and lipids was obtained and considered the most yield efficient as no-destructive technique with a total of 7% of the lipid extracted. The described effect was attributed to an increased permeability of the membranes, while the possible contribution of stress induction can be neglected because extraction was carried out straight after the treatment. Compared to other solvent-free extraction techniques, reversible electroporation was the only non-destructive method, and the resulting debris-free nature allows for a high degree of purity. Moreover, growth regeneration contributes to higher sustainability of the overall process.

The compounds synthesized and extracted from microalgae can find several applications. In particular, proteins and lipids, rich in omega-3 fatty acids, together with other compounds such as pigments, vitamins and polysaccharides can be used in the food sector (healthy food formulation, dietary supplements) but also in pharmaceuticals, cosmetics, nutraceuticals, aquaculture and biofuels one (Cuellar-Bermudez et al., 2015).

To the best of our knowledge, this is the only report on the application of reversible electroporation to microalgae for extraction purposes. The application of sustainable, energy efficient, and

simultaneous extraction methods for various compounds from microalgae or plants could be of great interest for the food industry and is, therefore, a very promising field for further investigation.

3. Potential and challenges for industrial implementation

The use of PEF as an advanced non-thermal processing method has received significant attention in the food industry due to increasing consumer demand for fresh-like food products. The PEF technology is considered a mild preservation and processing technology compared to its traditional alternatives, such as heat pasteurization or enzymatic extraction (Pataro & Ferrari, 2020; Huang & Wang, 2009). However, most industrial applications of PEF in the food industry focus on irreversible electroporation; which requires partial or total disintegration of cells.

Reversible electroporation has been studied for several different purposes in food products as described in this review. However, reversible electroporation is not currently used on an industrial scale due to several challenges. First, it is essential to apply a mild treatment to achieve the reversible effect of electroporation on cell membranes. If the threshold of reversible electroporation is exceeded, the process purpose fails (Benz & Zimmermann, 1981).

A serious challenge for the application of reversible electroporation at the industrial level lies in its detection and, in particular, in the differentiation between reversible and irreversible processes.

Various authors have previously described different methods to detect and quantify electroporation in microorganisms, food and biological tissues (García-Gonzalo & Pagán, 2016; Lebovka & Vorobiev et al., 2017; Napotnik & Miklavčič, 2018), reporting benefits and disadvantages of each. Among these methods, optical microscopy with or without the aid of several stains, electron microscopy for quantification of the morphological properties of membranes, measurements of electrical characteristics, such as electrical conductivity, dielectric constant and permittivity, measurement of diffusion coefficient, of texture, or acoustic tests have all been investigated for describing the electroporation effects. All methods are mostly destructive, affecting the structure of the material and therefore leading to some measurement error. These methods are not universal, since they measure different membrane modifications, so for a more accurate information, more than one method should be employed, increasing the complexity of the measurement. The processing of large volumes of product would also need the development of reliable methods for on-line monitoring of the efficiency of the process. Accurate quantification of the occurrence of reversible electroporation is more complex and not often explored in food matrices. It is important to perform uniform reversible electroporation throughout the food product such as fruits and vegetables, which are characterized by

heterogeneous structure, different tissue types, and the presence of air (Dymek et al., 2015; Dellarosa et al., 2018). In addition to the different cell structures and sizes, there is a different percentage of air content in most vegetables and fruits, and within the same product, the location of the air content varies in the different tissue types, which affects the overall conductivity of the matrix. Furthermore, the physiological characteristics (e.g., ripeness, structure, varietal variations, climatic conditions, postharvest stress) of the raw material may also influence its response to electric treatment; this is difficult to predict and control on an industrial scale setting. Treatment parameters, therefore, would need to be optimized to both achieve the effect of electroporation throughout the tissue and maintain cell viability after treatment. Studies on apples (Dellarosa et al., 2018) and microalgae (Luengo et al., 2014), confirmed that at low voltages both reversible and irreversible electroporation were observed on the same tissue after PEF treatment, due to the aforementioned high structural and physiological variability, leading to heterogenous electroporation. This issue represents a serious obstacle to the scale up of the applications of reversible electroporation presented in this review.

Similarly, various factors (e.g., such as species and strain, structure, composition and physical state of membranes and envelope, surface charge ecc) can influence the impact of treatment on microbial fermentation. Each microorganism is characterized by its own specific threshold for reversible electroporation, and its own specific metabolism which, in turn, can be influenced by the composition of the medium, pH, and temperature. Due to the interaction of numerous variables, the effect of reversible electroporation is complex to predict and cannot be simply generalized to meet the requirements of different applications.

The design of the treatment chamber for PEF application is another key aspect for achieving homogenous treatment through the flow of the raw material (Knappert et al., 2019). Research has mainly focused on the use of batch chambers in the laboratories, where a uniform electric field can be generated between two parallel electrodes in a closed chamber. However, in the food industry the large volumes usually need to be treated in continuous flow systems. In irreversible electroporation applications, this is not a problem because, above certain thresholds parameter values, it is possible to achieve homogeneous treatment of the entire tissue. It becomes an issue when reversible electroporation is the purpose of applying PEF in a continuous system. As examples, leafy vegetables such as spinach are processed in high volumes in the industry and require very large treatment chambers to match the industrial capacities. In a recent patent (WO 2021/107853 A1, 2021), an attempt was made to solve this issue by designing a chamber with two opposite electrode units of specific size and shape, placed behind at least one conveyor belt with perforation, so that the treatment

becomes homogeneous. The conveyor belts between the electrodes and the treatment substrate allow to avoid any hot spot occurring around the electrodes, which could cause non-uniform electroporation by the substrate passing in between.

Another challenge is to match the reversible PEF pre-treatment with the well-established and commonly applied operations in the food industry. For instance, drying of herbs is usually performed at high temperatures, around 100-120 °C. When reversible electroporation is applied prior to drying, the recommended drying temperatures are much lower (40 – 50 ºC) to maintain the effect of electroporation during drying. However, high temperature drying is mainly required for inactivating microorganisms. Therefore, it is difficult to use reversible electroporation widely in the dry herb industry. One possibility is to combine reversible electroporation with other drying techniques, for example microwave vacuum drying. This is a drying technique that takes a shorter time compared to freeze drying or conventional drying and is suitable for heat-sensitive products. To date, few studies have been conducted on the application of PEF prior to microwave vacuum drying (Nowacka et al., 2019); however, reversible electroporation has not been investigated as a prior to this drying technology.

At low production volumes and in a more controlled farming, it might be possible to apply reversible PEF prior to drying or to freezing, to obtain a higher quality product, with more aroma and colour. This could be interesting for premium products where quality is particularly important and the process is highly controlled, leading to a more standardized product, which in turn would make PEF optimization more efficient. Local small-scale producers are becoming increasingly important for sustainability reasons. Reversible electroporation of food products is one of the emerging technologies with unique offerings, especially for small scale producers. However, it still requires significant investment, and budgets for adapting new technologies are often limited in small-scale production. To make the technology affordable for small scale producers, PEF system suppliers could consider offering smaller scale power suppliers that could reduce the costs. Government and EU funding could also be considered as an option to encourage small business adoption of this emerging technology.

Food processing operations such as freezing, drying or fermenting are essential and commonly used in the food industry to extend shelf-life. As consumers become more health conscious, the demand for high quality food products is increasing. Undoubtedly, the food industry is undergoing a dynamic

process of change to meet the demands of an ever-growing global population. As the present review has shown, reversible electroporation offers various potential applications that could represent an interesting opportunity for the food industry. Therefore, to apply reversible electroporation on an industrial scale in the future, significant efforts and investments are still required, both in terms of process optimization research and equipment design. Investigations of reversible electroporation for industrial applications need to focus on increasing the value of the products compared to the energy/investment cost, particularly in these challenging times. It is well known that the application of reversible electroporation requires less energy input compared to irreversible electroporation, which is an advantage for low carbon footprint and enables sustainable applications in the food industry.

4. Conclusions and future perspectives

Interesting results have been obtained for different applications of reversible electroporation in food, however, the variability of the process conditions often does not allow to obtain clear indications of the optimal ranges, and process optimization is usually required for each specific target. The output of the treatment is bound to complex phenomena such as the metabolic response to PEF-induced stress, which has been only partially explored. Moreover, even when initial results are promising, the conditions required are, with the current state of the art, not suitable for industrial implementation (as in the case of improving freezing tolerance) or are limited to a small range of products (drying of leafy vegetables). Optimization of reversible electroporation parameters for all described applications requires further investigation. However, when technological challenges are overcome, reversible electroporation has various potential applications, representing an opportunity for innovation in the food industry.

To date, reversible PEF has not been implemented in the industry yet and the available literature is quite scarce, leaving many gaps to be filled. Moreover, from an examination of the published literature, it appears that the titles and keywords used very rarely directly reflect the use of the term “reversible electroporation or electroporation”. While it is very easy to find papers dealing with irreversible electroporation aimed at cold pasteurization or mass transfer, it requires more efforts to identify papers on reversible electroporation of food tissues, for any of the investigated applications. This may represent a limitation to further investigation on this promising technology and its implementation in the industrial environment. Probably, the use of ‘reversible electroporation’ or

'reversible permeabilization' as keywords, will allow a better distinction between the two processes and an easier identification of the relevant publications.

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Figure captions

Figure 1. Scheme of the mechanism leading to reversible or irreversible electroporation after the application of pulsed electric field.

Figure 2. Schematic representation of the various areas of application of PEF technology (adapted from Martin Gundersen's webpage, Pulsed Power Group, University of Southern California, http://www.usc.edu/dept/ee/Gundersen/. The figure is reused with permission from Martin Gundersen).

Figure 1

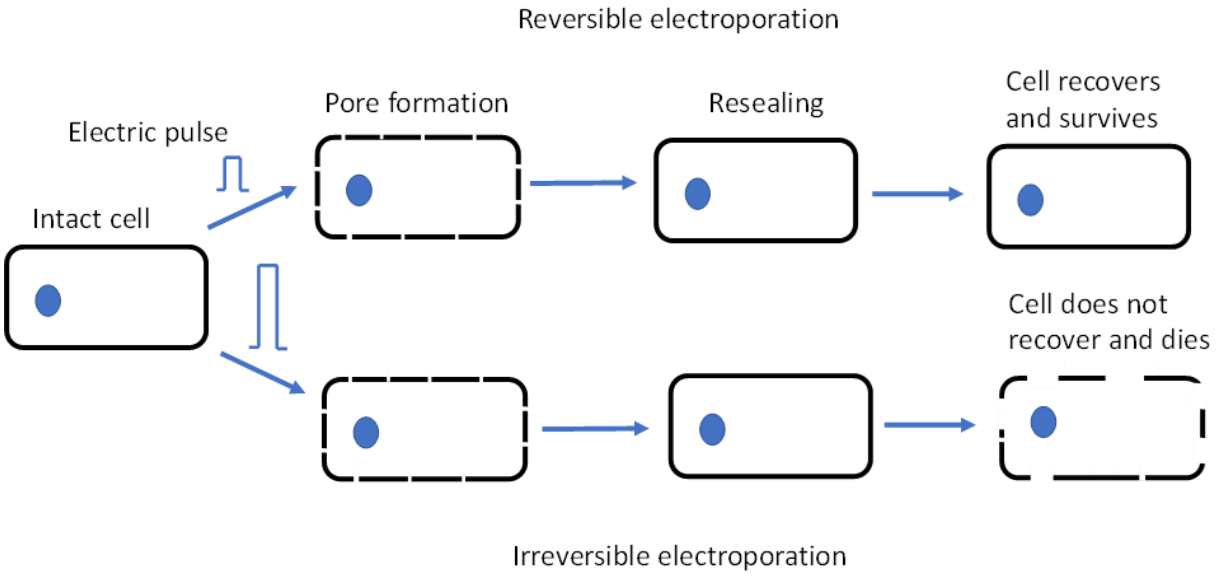

Figure 2

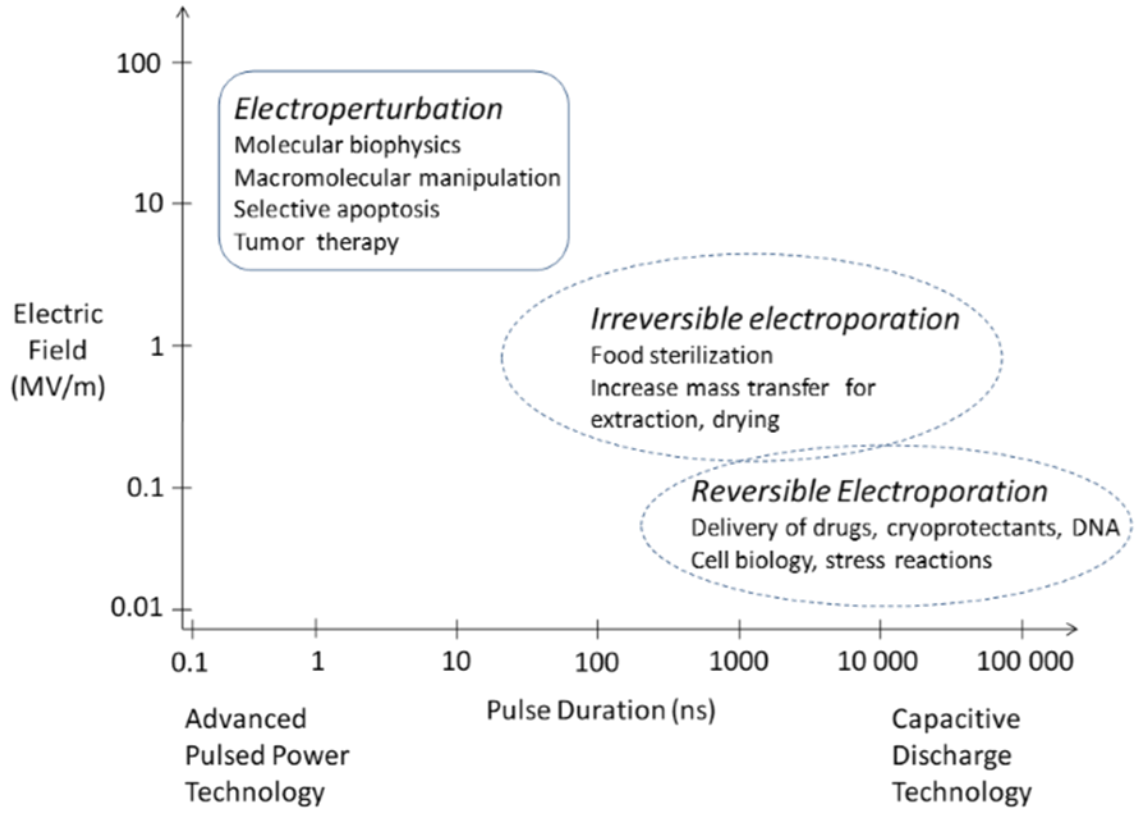

Table 1. Effect of reversible electroporation on the production of secondary metabolites in vegetable products and vegetable cell cultures

Product	**PEF treatment parameters**	**Main results**	**Reference**
		Vegetable products	
Pumpkin (*Cucurbita maxima*)	2 kV/cm 20 monopolar pulses of 4 μs, 0.1Hz	Higher polyphenols and carotenoids content after 24 h storage at 4°C	(García-Parra et al., 2018)
Tomato (*Solanum lycopersicum* L.)	40-200 kV/m, 5 monopolar exponential-wave pulses 0.1 Hz specific energy inputs (0.02 and 0.38 kJ/kg)	After storage at 4, 12 and 20°C for 1, 3 and 5 days, accumulation of carotenoids without negative effect on quality	(González-Casado et al., 2018)
Apple (*Malus domestica*)	i) 0.4 kV/cm, 5 pulses (0.01 kJ/kg, 20μs total treatment time); ii) 2.0 kV/cm, 35 pulses (1.8 kJ/kg, 140 μs total treatment time) and iii) 3.0 kV/cm, 65 pulses (7.3 kJ/kg, 260 μs total treatment time) Fixed parameters: 4 μs monopolar pulses, 0.1 Hz,	After 24 h at 22°C, the treatment at 0.4 kV/cm with 5 pulses promoted increase in selected polyphenols and quality attributes. Higher energies lead to irreversible damages	(Ribas-Agustí et al., 2019)
Olives, maize, soybeans (*Olea europaea*, *Zea mays*, *Glycine max*)	Range of 0.6–1.3 kV/cm, 120 pulses	Increase yield of oil in olives, increased recovery of isoflavonoids in soybean oil and phytosterols in maize germ oil.	(Guderjan et al., 2005)
Apple (*Malus domestica*)	0.4–2 kV/cm, using 5–35 monopolar pulses of 4 μs, 0.1 Hz, specific energy input of 0.008–1.3 kJ/kg.	Accumulation of phenolic compounds. Highest increase after 0.008 kJ/kg treatments and storage for 24 h at 22 °C. Highest increase in antioxidant capacity when storing fruits at 4 °C for 12 h.	(Soliva-Fortuny et al., 2017)

Tomato (*Solanum lycopersicum* L.)	0.4–2 kV/cm, 5–30 monopolar pulses of 4 μs, 0.1 Hz.	After 24 h at 4°C: increase of selected polyphenols, carotenoids and increased antioxidant activity, depending on the treatment intensity	(Vallverdú-Queralt, Odriozola-Serrano, et al., 2013; Vallverdú-Queralt et al., 2012; Vallverdú-Queralt, Oms-Oliu, et al., 2013)
Apple, berries, red grapes varieties (*Malus domestica*, *Vaccinium corymbosum*, *Ribes rubrum* *Vitis vinifera*)	0.3-4 kV/cm, variable number of pulses and duration, 1 Hz	Lower PEF intensities induced de novo synthesis of polyphenols	(Balaša, 2014)
Carrot (*Daucus carota*)	350 kV/m, 5 pulses (580 ± 80 J/kg)	Enhancement of phenolic content after 24 h due to stress induction	López-Gámez et al., 2020
	Cell cultures		
Grape (*Vitis vinifera*)	1.6 kV/cm, 10 pulses plus Etephon addition 28 mg/L	PEF elicits defence response and stimulates the accumulation of polyphenols and anthocyanins.	(Cai et al., 2011)

Grape (*Vitis vinifera*)	1.6 kV/cm, 10 monopolar exponential-decay pulses, 1 Hz, total specific energy: 0.32 J/kg	Combination with etephon stimulated anthocyanins synthesis during 14 d after treatment	(Saw et al., 2012)
Tomato (*Solanum lycopersicum* L.)	0.600 and 1.200 kV/cm, 1 and 9 pulses, or 900 V/cm, 5 pulses	0, 4, 96 h after PEF application: Phenylalanine ammonia lyase activity increased with the increase in the strength and number of pulses of PEF application Maximum activity found for 9 pulses at 1200 V/cm, 4 h after treatment	(Gürsul et al., 2016)
Apple and grape (*Malus domestica, Vitis vinifera*)	0.2-2.4 kV, 20-100 pulses of 6 μs, 1-2 Hz	Dose dependent accumulation of polyphenols. Increased phenylalanine ammonia lyase activity and alteration of polyphenol oxidase activity	(Balaša, 2014)
Chinese yew (*Taxus chinensis*)	1 kV/cm 20 μs pulses, 50 Hz	Up to 20 days: significant intracellular accumulation of secondary metabolites	(Ye, Huang, Chen, & Zhong, 2004)
Rocket *(E. sativa)* hairy root cultures	2.5 or 5 kV/cm (τPuls 150 μs), 1 or 2 exponential decay pulses in combination with Jasmonic acid (JA) 50 μM	Combination of the chemical elicitor JA with PEF: increased total glucosinolates content compared to the control, and cultures treated with only PEF or JA, 24 h after treatment.	(Kastell et al., 2018)

Table 2. Similarities and differences observed among studies related to reversible electroporation aimed at secondary metabolites production on the same food matrix

Product	Similarities	Differences	Comments
Apple	Low applied energy (<0.4 kV/cm) promotes the accumulation of phenolic compounds Increased metabolites are analysed always 24 hours after PEF application The same cultivar was considered in all the studies (Malus domestica cv. Golden Delicious)	Temperature of storage are often different (4, 16 or 22 °C) Only Ribas-Augusti et al 2019 determined phenolics by HPLC methods, all the other using spectrophotometric determinations Only Balasa et al., (2004) determined PAL activity and membrane permeabilization (CDI)	PEF-induced stress responses are the direct responsible for the described results. Phenolic compounds are recognized as molecules involved in stress protection in plants (Samec et al., 2021). However, to make sure that the effect is related to de novo synthesis, enzymatic activation should be determined (e.g., PAL).
Tomato	Increased accumulation of carotenoids. Increased metabolites are analyzed by all authors after 24 hours at 4°C after PEF application	Different cultivars were used, accumulation of carotenoids tested on raw tomatoes (González-Casado et al., 2018) or on juice (Vallverdú-Queralt, Odriozola-Serrano, et al., 2013; Vallverdú-Queralt et al., 2012; Vallverdú-Queralt, Oms-Oliu, et al., 2013)	The result is attributed to increased production due to metabolic stress. Gonzales-Casado et al 2018, observed an increase in respiratory activity together with an increase of pH and soluble solids that could be indicating a metabolic change, however, tissue damage was also observed, therefore it is difficult to determine which effect was responsible for carotenoids increase.
Cell cultures	Accumulation of secondary metabolites due to de-novo synthesis	Mostly different cell cultures used, with different PEF protocols. Different secondary metabolites are investigated in different studies (e.g., polyphenols, anthocyanins, glucosinolates). Different storage time after PEF (from few hours to 20 days)	Few studies include investigation on enzymatic activation, however increased accumulation of secondary metabolites is observed over long storage, which suggests an increased production. Due to differences in PEF protocols, it is difficult to obtain clear indications on the optimal parameters.

	Two studies investigated the combination with chemical elicitors (JA and etephon)	Many studies investigate only 1 or 2 PEF conditions, effect of different parameters is not investigated

Table 3. Effect of reversible electroporation on seed germination

Product	PEF treatment parameters	Main results	Reference
Barley seeds (*Hordeum vulgare*)	0-1.200 kV/cm, 50 rectangular pulses of 1 ms, spaced by 2 ms	Impairment of radicle growth, no significant effect on the seeds' gross metabolic activity. No effect on protein pattern; decrease of α-amylase concentration	(Dymek et al., 2012)
Arabidopsis thaliana seedlings	5-50 kV/cm, 10-100 no of pulses, 10-100 ns pulses. Specific energy: 0.1-10 kJ/kg	Growth stimulating effect after short pulse exposition at 5 kV/cm and 0.1 kJ/kg	(Eing et al., 2009)
Wheat seeds (*Tritium aestivum L.*)	2, 4, and 6 kV/cm for (25 and 50 pulses) pulse width of 100 μs, 1 Hz. T<30°C Specific energy: 1.5-7.5 kJ/kg	Increased water uptake, germination and growth parameters at 6 kV/cm. Significant changes in metabolism, enhanced growth, increased activity of antioxidant enzymes, increased protective capacity of harvested shoots	(Ahmed et al., 2020)
Wheatgrass (*Triticum aestivum*)	0.5, 1.4 and 2 kV/cm, 100 pulses, width of 20 μs, 5 Hz. T: 20°C	PEF treatment of seeds at field strengths of 1.4 kV/cm or less did not influence seed germination. However, seedlings produced from seeds treated at an electric field strength of 1.4 kV/cm were slightly larger than seedlings from untreated seeds. In contrast, PEF treatment of seeds at 2 kV/cm reduced coleoptile and primary leaf growth by at least 6 mm and 10 mm respectively, as compared to seedlings from untreated seeds.	(Leong et al., 2016)
Haloxylon ammodendron Seeds	10, 20 and 30 kV/cm, 20 pulses, width of 100 ns	Significant effect on seed germination and pre-growth due to NO generation and a reduction in the oxidation-reduction potential (ORP) after nsPEFs-seeds treatment	(Su et al., 2015)

Arabidopsis thaliana seedlings	5-20 kV/cm, 100 pulses, width of 10 ns, 5 Hz Specific energy: 0.25-4 kJ/kg	Growth effect of nsPEF was found dependent on pulse characteristics. The growth can be enhanced effectively by choosing appropriate pulse intensity	(Songnuan & Kirawanich, 2012b)
Wheat grains (*Tritium aestivum L.*)	Energy range: 1.7-17.28 J, monopolar rectangular pulses, treatment time range: 2.47-19.78, Frequency range: 100- 180 Hz	PEF processing of the wheat seeds improved vigor, promote cold and salt stress tolerance, and inactivated surface microflora	(Akdemir Evrendilek et al., 2021)

Table 4 . Similarities and differences observed among studies related to reversible electroporation aimed at seed germination on the same food matrix

Product	Similarities	Differences	Comments
Arabidopsis thaliana seedlings	nsPEF applied. Enhancement of growth observed at lower voltages applied.	Seedling growth monitored for different length of time (7-14 days) and different temperature/light conditions.	
Cereal seeds (wheat and barley)		Completely different pulses protocols applied by each author, including use of pulses of ms (Dymek et al., 2012) and µs (Leong et al., 2016; Ahmed et al., 2020) length. Different parameters measured as response. Effects monitored for different length of time after PEF (from 8 h to 8 days)	Some authors (Leong et al., 2016; Ahmed et al., 2020) agree that PEF treatment above 1.4 kV/cm stimulated the metabolic activity of different types of seeds due to the response to increased ROS production, however the actual ROS production was not analysed.

Table 5. Effect of reversible electroporation on microbial fermentation

Product	PEF treatment parameters	Main results	Reference
S. cerevisiae in YEPG medium	0.5 to 1.5 kV/cm, bipolar square pulses of 20 µs, total length of pulses: 8 ms	100% increase of cell growth at 0.85 kV/cm field strength.	(Fologea et al., 1998)
Lactococcus lactis subspecies *cremoris* in Oxoid M17 Bros medium	8 kV/cm, 200 pulses and pulse-length of 1 µs	PEF increased exopolysaccharide yield when applied as one-pass treatment (32%) and as a circular treatment for 4 h (94%). PEF led to lower molecular weight EPS	(Ohba et al., 2016)
S. cerevisiae in YPD medium	double-spiral electrode reactor, 2-4 kV, average 8.3 mm interelectrode distance, flow rate 160 ml/min,	PEF treatment induced the expression of oxidation stress response genes, and glutathione played an important role in the stress resistance induced by PEF	(Tanino et al., 2012)
Aspergillus niger in Basal medium	0.57-2.85 kV/cm 1-20 ms pulse duration 0.1-10 Hz frequency	The yield of citric acid production: -did not change at different pulse duration 1-20 ms. -increased with highest Electric field at 2.854 kV/cm -maximized at 1Hz (1.4-fold compared to control)	(Fiedurek, 1999)
Streptomyces avermitilis in YMS medium	nsPEF 5, 10, 20 kV/cm– 20 pulses 30 kV/cm– 100 pulses	Cell viability increased after 20 pulses 10 kV/cm. Proliferation rate increased after 20 pulses 20 kV/cm. Avermectin production time reduced from 7 to 5 days. Oxidation-reduction rate decreased with PEF. Gene expression enhanced with PEF.	(Guo et al., 2016)
Lactobacilli (*L. acidophilus; L. bulgaricus; L. case*i) in MRS medium	2.5-5.0-7.5 kV/cm 3-3.5 or 4 ms treatment time	Increased growth of lactobacilli cells, increased uptake of cholesterol into cell membrane after electroporation permeability	(Lye et al., 2011)

S. cerevisiae in Sabouraud agar medium	0.020-2.000 kV/cm bipolar pulses 1-10000 no of pulses 1-10 no of trains 10 µ s pulses 10 ms repetition time 10 s space between trains	PEF increased the yeast viability in two ways: "logarithmic" and "saturated". At logarithmic electrostimulation, the yeast cells viability increased with the increased E. field and longer fermentation times (24h), while at saturated electrostimulation, the viability of yeast cells was higher for short time fermentation (1h).	(Mattar et al., 2014)
S. cerevisiae suspension in water	0.100 and 6.000 kV/cm Monopolar pulses 1000 pulses,100 µs pulse duration 100 ms pulse repetition time 18 µS/cm conductivity	Fermentation process was enhanced with PEF. Sugar consumption was increased with PEF. 30% more mass reduction was reached with PEF treated samples after fermentation. Same mass reduction required extra 20 h in control samples.	(Mattar et al., 2015)
Kluyveromyces marxianus IMB3	0.625 - 3.750 kV/cm 10 ms	Ethanol production from cellulose increased by 40% with application of PEF. By increased E. field, ethanol production was increased but not as much as when using 0.625 kV/cm.	(McCabe et al., 1995)
L. acidophilus and *L. delbrueckii* ssp. *Bulgaricus in MRS medium*	1 kV/cm, positive square unipolar pulse width: 3 µs, pulse period: 0.5 s, electric field strength: delay time: 20 µs, flow rate: 60 mL/min, T: 40.5°C	Improved acid tolerance, exponential growth, and protease activity of both studied strains	(Najim & Aryana, 2013)
S. cerevisiae	Optimized parameters 3 kV/cm, 10 µus pulse width, 1 Hz, Total exposure time 10 min	PEF enhanced the accumulation of selenium and zinc in yeast cells.	(Pankiewicz et al., 2017)

L. rhamnosus B 442	Electric field strength in the range 0.1- 6.0 kV/cm, pulse width: 20 μs, frequency: 1 Hz, exposition time: 15 min	The optimal parameters (3.0 kV/cm) promoted the highest bioaccumulation of zinc (500 μg/mL medium)	(Góral et al., 2019)
Lactobacilli and Bifidobacteria strains in MRS medium	2.5, 5.0, 7.5 kV/cm 3, 3.5, 4 ms treatment time	Increased cell membrane permeability and membrane lipid peroxidation. Enzyme activity is increased with higher production of aglycones.	(Yeo & Liong, 2013)
Bifidobacterium longum in MRS medium	7.5 kV/ cm 3.5 ms treatment time	Increased viability, intracellular and extracellular β-glucosidase activity, leading to enhanced production of bioactive isoflavone aglycones in mannitol soymilk. Probiotic potentials (tolerance toward acidic and intestinal bile salt condition and antimicrobial activity toward pathogens) also enhanced	(Yeo et al., 2014)
Lactobacillus strains in MRS medium	Field strengths: 2.5, 5.0 and 7.5 kV/cm Pulse durations: 3, 3.5and 4 ms	7.5 kV/cm for 3.5 ms: enhanced β-glucosidase activity leading to increased bioconversion of isoflavones glucosides to aglycones in biotin–soymilk	(Ewe et al., 2012)
Chlorella vulgaris in MRS medium	Various treatment with parameters in the following ranges: E: 10.5-19.97 kV/cm, pulse number: 1.83-15.88, pulse width: 25-100 ns, f: 3-20 Hz, treatment time: 0.61 s, Specific energy input: 217-507 J/Kg	Longest pulse width (100 ns) resulted in the highest biomass yield nsPEF treatments enhance cell proliferation based on intracellular and plasma membrane-related effects.	(Haberkorn et al., 2019)
L. plantarum in MRS medium	E: 40-60 kV/cm, number of pulses: 100-600, Pulse width: 35 ns, f: 1-50 Hz, applied during the log growth phase of the bacteria	Metabolism of lactic acid bacteria was positively stimulated by the nsPEF treatment 19% increase in L-lactic acid, 6.8% increase in D-lactic acid and 15% increase in acetic acid observed over control.	(Kanafusa et al., 2021)

		Increased levels of metabolites dependent on the applied voltages	
Hanseniaspora sp. Strain in YPD medium	0.285 kV/cm, N trains of n=10 pulses. Pulse duration: 100 µs, pulse period time: Δt =1 ms. Time between trains: Δt =1 s 1) PEF-treatment for 12 h from the beginning of fermentation including the lag phase, 2) PEF-treatment for 12 h during the log phase (after 12 h of fermentation), 3) fermentation of the medium inoculated with treated pre-culture during 6 h (during the lag and the log phases), 4) fermentation of the medium treated for 12 h during the log phase (after 12 h of fermentation), which was inoculated by a pre-culture previously PEF treated for 6 h	Yeast concentration and biomass yield increased significantly for all the treatments. Increased growth rates were accompanied by decreased ethanol rates and contents The highest alcohol reduction was observed when PEF treatment applied to pre-culture for 6h. Yeast cells response to PEF treatment changes with time: higher biomass yield during the first hours of fermentation	(Al Daccache, Koubaa, Salameh, et al., 2020)
Hanseniaspora sp. Yeast in YPD medium	Intensity in the range 0.072–0.285 kV/cm. during the different fermentation phases (Lag, exponential and log phases)	Moderate PEF stimulate Hanseniaspora sp. yeast reducing fermentation time and increasing biomass concentration. Maximal yeast growth rate at 285 V/cm applied during both Lag and early exponential phase, and Log phase. Together with a faster consumption of glucose in the medium during the fermentation. Response of yeast: more biomass increases during the Lag and early exponential phase than the Log phase.	(Al Daccache, Koubaa, Maroun, et al., 2020)
Arthrospira platensis SAG	10.23 kV/cm, pulse number: range 0.23-2.96, 100 ns, Frequency: range 1-13 Hz,	increased cell proliferation, detectable after repeated nsPEF treatment in the exponential growth phase. increase in pigments was detectable.	(Buchmann et al., 2019)

21.99 in Zarrouk medium	Proteomic analysis revealed a possible stress response pathway.

Table 6. Similarities and differences observed among studies related to reversible electroporation aimed at enhancement of fermentation on the same food matrix

Product	**Similarities**	**Differences**	**Comments**
S. *cerevisiae*	Stimulation of fermentation kinetics	PEF applied to the microorganism in different growth media; different indirect effects were evaluated (e.g., mass growth, yeast viability, sugar uptake, accumulation of metal ions)	
Lactobacilli	Increase in cell growth, increased production of specific compounds after process optimization with increased health promoting properties	Completely different PEF protocols (also involving nsPEF). Authors observed different parameters related to fermentation kinetics and products	Authors often observed the occurrence of irreversible electroporation, but the surviving cells showed higher viability and enhanced growth, attributed to enhanced uptake of nutrients

Table 7. Effect of reversible electroporation on induction of freezing tolerance of vegetable tissues

Product	PEF treatment parameters	Main results	Reference
Spinach (*Spinacia oleracea*)	580 V/cm, 10 trains of 25 bipolar pulses of 25 µs	Combination of PEF and Vacuum Impregnation (VI) in 40% trehalose allowed to preserve cell viability after freezing and thawing	(Phoon et al., 2008)
Spinach (*Spinacia oleracea*)	Material: Trehalose impregnated leaves 400 V/cm, 50 monopolar pulses, 250 µs pulse duration, 600 µS/cm conductivity	PEF induces a metabolic response that may increase resistance to abiotic stress	(Dymek et al., 2016)
Spinach (*Spinacia oleracea*)	700 V/cm, 2 train of 500 bipolar pulses of 200 µs (500Hz)	Improved freezing tolerance when combined with VI with cryoprotectants and cold acclimatation	(Demir et al., 2018)
Strawberries (*Fragaria ananassa*)	850 V/cm, 5 Bipolar pulses of 100 µs, specific energy input: 213 J/kg	PEF combined with VI allowed to maintain cell viability and improved colour characteristics after freezing and thawing. PEF improved the number of viable cells compared to only VI.	(Velickova et al., 2018)

Table 8. Similarities and differences observed among studies related to reversible electroporation aimed at freezing tolerance improvement production on the same food matrix

Product	Similarities	Differences	Comments
Spinach	Combination of PEF with VI with cryoprotectants increases freezing tolerance. Trehalose is used in all studies as cryoprotectant.	Different PEF parameters are used, including mono and bipolar pulses. Concentration of trehalose varying between 11 and 40%. Method for freezing not the same (liquid nitrogen or blast freezer). Dymek et al., (2016) investigated only the metabolic consequences and not the survival after freezing	The combination with a cold stress prior to freezing can improve the results, however it is not applicable in industrial environment. Even if it is an important contribution to the field, the results can be regarded as preliminary. In depth investigations of the relation between PEF parameters and stress-induced cross tolerance as well as the search for potential metabolic markers that can be used for process optimization are missing

Table 9. Effect of reversible electroporation on drying through opening of stomata

Product	PEF treatment parameters	Main results	Reference
Basil (*Ocimum basilicum*)	EF: 600-1500 V/cm, 65 monopolar pulses varying duration: 120-150 µs, pulse space: 500/760 µs,	Reversible and irreversible PEF treatment shortened the drying time (37%, drying at 50°C). Reversible PEF: dried product with improved rehydration capacity, colour and aroma retention compared with non-permeabilized leaves and the leaves treated with irreversible electroporation.	(Kwao et al., 2016)
Basil (*Ocimum basilicum*)	650 V/cm, 65 monopolar pulses, duration: 150 µs pulse, 760 µs pulse space	Reversible PEF treatment shortened the drying time with all three different drying methods: air drying (57%, at 40C), vacuum drying (33%) and freeze-drying (25%). Effect of reversible electroporation higher at lower air-drying temperatures.	(Telfser & Gómez Galindo, 2019)
Thai Basil *(Ocimum basilicum var. thyrsiflora)*	PEF 1 (650 V/cm, 200 pulses, 50 µs width, 27.46 kJ/kg specific energy input) PEF 2 (650 V/cm, 125 pulses, 175 µs width, 60.07 kJ/kg specific energy input) PEF 3 (650 V/cm, 150 pulses, 50 µs width, 20.60 kJ/kg specific energy input) MEF (100 V/cm, 1200 Hz frequency, 1200 ms total treatment time, 20.23 kJ/kg)	Guard cells electroporation occurs at higher number of pulses (PEF 1 and PEF2) MEF protocols did not show any significant reduction in drying time (no electroporation of guard cells) Drying time PEF1<PEF2<PEF 3, MEF, control Only monopolar pulses electroporate stomatal guard cells.	(Thamkaew & Gómez Galindo, 2020)

Table 10. Similarities and differences observed among studies related to reversible electroporation aimed at drying through stomatal opening on the same food matrix

Product	Similarities	Differences	Comments
Basil	PEF parameters were selected to obtain homogenous electroporation of guard cells on the surface of basil leaves.	Two of the studies have considered the drying kinetics and focused on the quality of the final product by using only monopolar pulses (Kwao et.al.,2016, Telfser & Gómez Galindo, 2019), while one of the studies have focused on optimization of PEF parameters to obtain homogenous electroporation of guard cells (Thamkaew & Gómez Galindo, 2020) and tried bipolar pulses and included MEF in the study. Telfser & Gómez Galindo also compared different drying techniques after application of reversible electroporation.	Implementation at industrial scale could only be possible if the issue of the microbial safety of big volumes of raw material can be solved without the need of drying the product at high temperatures. This solution needs to be cheap and easily implemented.

Figure 1

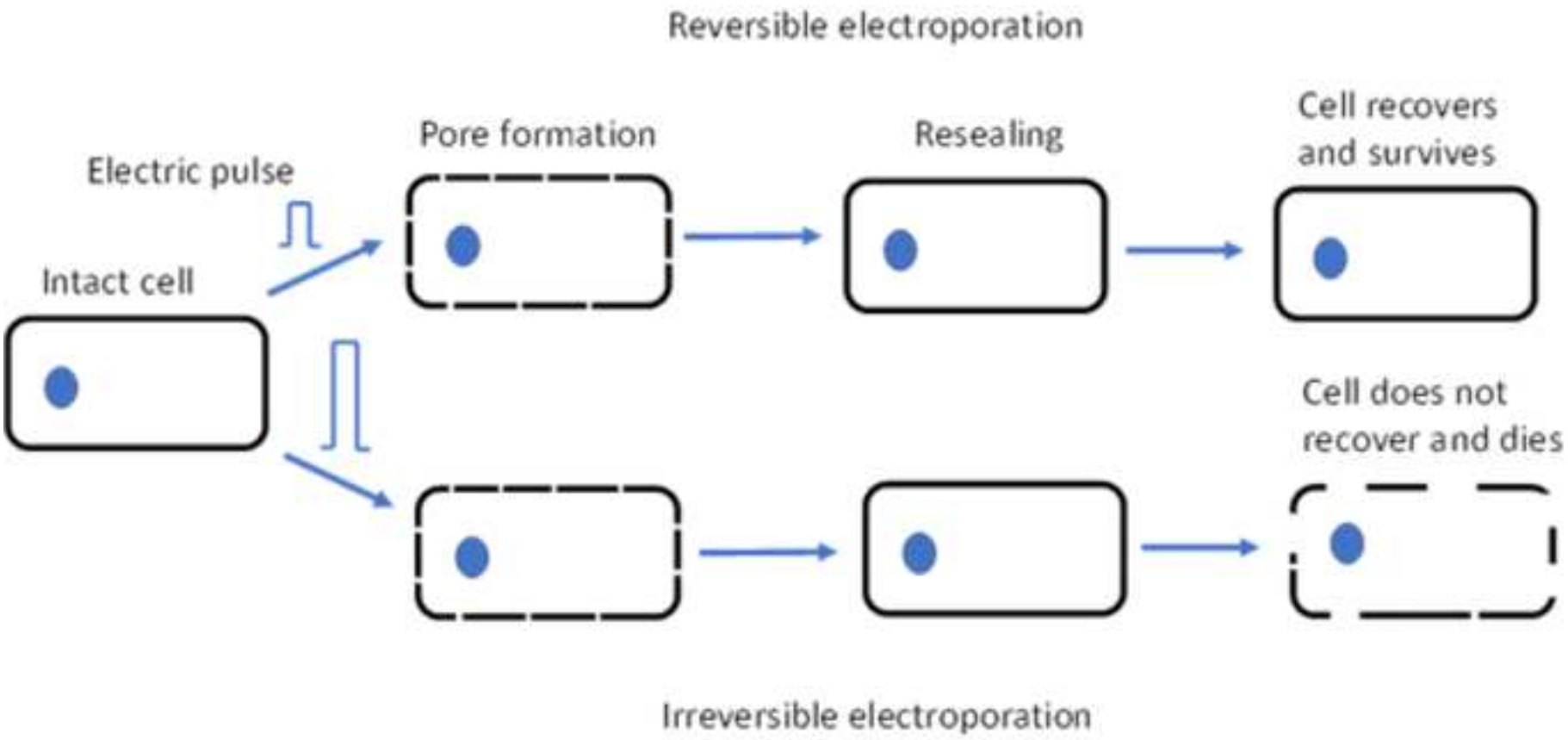

Figure 2