Application of Pulsed Electric Fields in Algal and Bacterial Cocultures: Investigating Bacterial Responses and Purity of Algal Protein Extract

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Abstract

Introduction: Pulsed electric field (PEF) technology shows promise for microbial control in biorefinery applications. However, its effectiveness in mixed cultures remains poorly understood. This study investigated the differential effects of PEF treatment on bacterial inactivation and algal protein preservation in a coculture of *Chlorella vulgaris* algae and *Delftia* sp. bacteria.

Methods: Algal and bacterial viable cell counts and viability were quantified using flow cytometry with differential fluorescent staining (SYTO 9, YO-PRO-1, fluorescein diacetate [FDA]), bacterial growth was monitored spectrophotometrically at 600 nm, protein extraction was determined by modified Lowry assay, protein profiles analyzed by SDS-PAGE, and extract antimicrobial activity was assessed by agar diffusion and growth inhibition assays. We compared PEF treatments at two energy levels (4 and 100 J/mL) against high-pressure homogenization (HPH) as a control, with assessments at different growth phases (days 1, 3, and 7).

Results: While PEF consistently inactivated >95% of algal cells, regardless of the growth phase, bacterial inactivation varied significantly, with maximum susceptibility on day 3 (70–80% mortality) when bacteria entered the starvation phase. Unexpectedly, on day 7, PEF treatment of cocultures led to bacterial proliferation, with viable counts increasing up to 4-fold compared with untreated controls. Analysis of algal extracts showed no antimicrobial activity against bacteria, and instead supported bacterial proliferation, suggesting that cellular disruption releases compounds that can be metabolized by surviving bacteria. Furthermore, while PEF preserved the integrity of algal protein profiles regardless of bacterial presence, HPH treatment of cocultures introduced a novel $\sim$27 kDa protein band, suggesting bacterial contamination of the extract.

Conclusion: These findings reveal the complex, growth phase-dependent dynamics inherent in PEF treatment of mixed microbial systems and provide critical insights into biorefinery applications in which microbiological control and product quality must be balanced.

Keywords: pulsed electric field (PEF), *Chlorella vulgaris*, microalgae-bacteria coculture, selective cell disruption, protein extraction, biorefinery, *Delftia* sp., nonaxenic cultivation

Introduction

Pulsed electric field (PEF) technology has emerged as a versatile tool across various sectors including medicine, environmental science, and food technology.[1–4] The growing interest in PEF stems from its advantageous characteristics, such as rapid processing times, elimination of hazardous solvents, nonthermal nature, and scalability potential.[5–8] PEF operates by establishing a potential difference across cell membranes, inducing pores that vary in size and persistence based on electrical parameters (voltage and pulse duration), and specific characteristics of the targeted cells (species, environmental context, etc.).[9–12] For microalgae, PEF treatments at higher electric field strengths enhance cell permeability, thereby enabling a number of applications including genetic modifications, eradication of toxic blooms, and extraction of valuable compounds.[13–15]

In recent years, PEF has gained attention for biomass processing in biorefineries. The integration of PEF into a wet biorefinery cascade presents significant advantages by bypassing energy-intensive drying steps and enabling sequential extraction of multiple valuable compounds. A key feature of PEF-assisted extraction is the post-treatment incubation period, which facilitates cell autolysis and release of intracellular

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components into the aqueous phase. During this incubation step, enzymatic processes drive protein release from various cellular compartments.[16] In the case of *Chlorella vulgaris*, a cell death-inducing factor (CDIF) is released that kills intact algal cells, reducing the specific energy requirement for protein extraction to a minimum of 3 J/mL.[17]

However, a critical knowledge gap exists in understanding the effectiveness of PEF in real-world biorefinery applications, where axenic monocultures are rarely achievable. Large-scale algal cultivation systems typically contain mixed microbial populations, either by design or through environmental contamination. In fact, algae-bacteria interactions can be beneficial, with certain bacterial species providing essential compounds that enhance algal growth and metabolite production. For instance, *Delftia* species recently identified in *C. vulgaris* bioreactors have been shown to stimulate algal growth under specific cultivation conditions.[18]

The transition from laboratory monocultures to industrial mixed cultures raises important questions regarding PEF treatment efficacy, product quality, and safety. Previous research has established that bacteria typically require higher specific treatment energies for inactivation than microalgae[19] and that microbial susceptibility to PEF varies with growth phase and cell concentration.[20,21] However, a significant gap remains in our understanding of how these differential responses emerge in coculture systems and their implications for downstream processing and product quality.

To address this knowledge gap, we investigated several interconnected aspects of PEF application in mixed cultures. We examined how PEF treatment differentially affected algal and bacterial viability in coculture systems across different growth phases, providing insights into the selective nature of electrical disruption. We also assessed whether the post-PEF incubation period, which is essential for protein extraction, influences bacterial survival, and potential contamination, which have direct implications for process design. Additionally, we evaluated whether bacterial presence affected algal protein extraction efficiency and profile integrity following PEF treatment, assessing protein extraction outcomes in mixed-culture systems. However, this study was limited to laboratory-scale investigation of PEF treatment effects on microorganism viability. Detailed protein characterization (including purification, identification, and biochemical analysis), long-term evolution of mixed cultures, large-scale applicability, and cost analysis were beyond the scope of this investigation.

By systematically addressing these questions, this study aimed to provide critical insights into optimizing PEF applications in mixed-culture biorefineries, where balancing microbial control with product quality is essential for commercial viability.

Methodology

Coculture of bacteria and microalgae

Chlorella vulgaris algae (SAG strain 211–12, University of Göttingen) were cultured in Tris-acetate-phosphate (TAP) medium (17.49 mM acetic acid; 19.98 mM TRIS; 7.01 mM NH_4Cl; 0.4 mM $MgSO_4 \times 7H_2O$; 0.34 mM $CaCl_2 \times 2H_2O$; 0.63 mM K_2HPO_4; 0.4 mM KH_2PO_4; 0.17 mM EDTA; 0.18 mM H_3BO_3; 0.08 mM $ZnSO_4 \times 7H_2O$; 0.03 mM $MnCl_2 \times 4H_2O$; 0.02 mM $FeSO_4 \times 7H_2O$; 0.007 mM $CoCl_2 \times 6H_2O$; 0.006 mM $CuSO_4 \times 5H_2O$; 0.001 mM $(NH_4)_6Mo_7O_{24} \times 4H_2O$ pH 7.0[22] for 7 d until stationary growth phase was reached under conditions described in.[17] *Delftia* sp. bacteria, previously isolated and identified from *C. vulgaris*-inoculated bioreactors,[18] were cultivated aerobically in tryptic soy broth (TSB, peptone from casein 17.0 g/L, peptone from soybean 3.0 g/L, D-(+)-glucose 2.5 g/L, NaCl 5.0 g/L, K_2HPO_4 2.5 g/L) for 24 h at 30°C.

For the coculture experiments, sterile flasks containing TAP medium were inoculated with axenic microalgae (initial optical density 0.1 at 750 nm) and bacteria (initial optical density 0.01 at 600 nm). As controls, additional flasks containing monocultures of either algae or bacteria were prepared in parallel. All flasks were maintained under algal culturing conditions without CO_2 supply for 7 d.

PEF processing

Cell suspensions were treated using a continuous-flow PEF treatment chamber as described previously.[17] In brief, a custom-built transmission-line pulse generator was used to deliver rectangular pulses with a duration of 1 μs and an amplitude of 8 kV. The treatment chamber consisted of a polycarbonate housing equipped with two plane-parallel stainless-steel electrodes separated by a 2 mm gap. Two distinct protocols were used for this study. The first protocol was performed under aseptic processing conditions to assess viability after PEF treatment. The cell suspension (100 mL) was transferred to a sterile sealed 250 mL flask and connected to the inlet of the PEF treatment chamber. The suspension was pumped through the PEF treatment system using a peristaltic pump (Cole-Parmer GmbH, Germany) with an outlet connected to a sterile receiving flask. A transmission-line pulse generator delivered square pulses using the following parameters:

Condition	*t (μs)*	*E (kV/cm)*	*f (Hz)*	*Φ (mL/s)*	*ΔW (J/mL)*
PEF 1	1.0	40	0.2	0.05	4.0
PEF 2	1.0	40	5.0	0.05	100.8

These energy levels were selected to investigate distinct biological endpoints in mixed cultures. The 4 J/mL treatment represents the minimum energy required for efficient *C. vulgaris* protein extraction, as previously established.[16,17] In contrast, bacterial inactivation typically requires substantially higher energies (40–175 J/mL) to achieve >2 $\log_{10}$ reductions (>99%).[19,23,24] The 100 J/mL treatment was selected as an intermediate energy level that exceeds established bacterial inactivation thresholds while avoiding the protein oxidation and aggregation documented at higher PEF intensities.[25–27]

The second protocol was performed out under nonfully aseptic conditions to assess the protein extraction. The coculture was first concentrated to 5 g/L by centrifugation (10,000 *g* for 5 min). The biomass obtained was then subjected to PEF treatment using the parameters described above. As a positive control, the biomass was subjected to high-pressure homogenization (HPH) using an EmulsiFlex-C3 homogenizer (Avestin, Canada) at a pressure of 1500 bar for 5 passes, with continuous cooling on ice.

The conductivity of the suspension was not adjusted and varied according to the day of cultivation (from 2.2 mS/cm at the beginning of cultivation to 1.2 mS/cm at the end of cultivation).

Populations changes analysis

We employed differential staining methods to distinguish and quantify the algal and bacterial populations. Samples were stained with either SYTO® 9 (5 mM, cell-permeable nucleic acid dye for total cell counts, Invitrogen by Thermo Fisher Scientific), YO-PRO-1® (1 mM, cell-impermeable nucleic acid dye for membrane-compromised/dead cells, Invitrogen by Thermo Fisher Scientific) or fluorescein diacetate (FDA, nonfluorescent, cell-permeable esterase substrate that indicates metabolic activity in viable algal cells, Invitrogen by Thermo Fisher Scientific). Briefly, samples were mixed with SYTO 9 (final concentration 0.5 μM in DMSO), YO-PRO-1 (0.1 μM in DMSO) or FDA (0.5 μM in DMSO) and incubated for 5–10 min in the dark. After incubation, stained samples were diluted by a factor of 10 in sterile TAP medium, and the fluorescence signals of the dyes were quantified using a flow cytometer (Attune™ Nxt, Thermo Fisher Scientific). Algal cells were distinguished from bacteria based on chlorophyll-a autofluorescence, and a number of events were recorded as described previously.[18] The data obtained were analyzed to determine (1) algal viability (%), calculated as the ratio of viable algal cells to the total number of algal cells; (2) bacterial viability (%), determined using the same approach; and (3) viable bacterial concentration, quantified by subtracting the number of dead bacteria from the total bacterial cell count.

Algae extract antibacterial assay

The antibacterial activity of microalgae extracts processed through PEF or HPH was assessed after an additional 24 h of incubation using the agar well diffusion method.[28] Tests were conducted using *Delftia* sp. and *Escherichia coli* (DSM 498) as target organisms. Furthermore, the antimicrobial susceptibility of *Delftia* sp. to microalgae extract was determined by inoculating bacteria into cell debris-free microalgae extract, which was maintained under algal cultivation conditions. Bacterial response was continuously monitored spectrophotometrically (at 600 nm) throughout the 3-d recultivation period.

Algae protein extraction determination

The soluble protein concentration in the sample supernatant was quantified using a modified Lowry Assay protocol (Detergent Compatible, DCTM, BioRad, Munich) according to the manufacturer's instructions. PEF-treated samples were incubated for an additional 24 h prior to analysis, whereas HPH-treated samples were analyzed immediately after processing. Protein concentrations were determined against a bovine serum albumin (Thermo Fisher Scientific, USA) standard curve and expressed as a percentage of cell dry weight ($\%_{CDW}$). Qualitative protein analysis was performed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to protocols described in,[16,29] with gels processed and analyzed using GelAnalyzer 23.1.1 software.

Statistical analysis

This study comprised three complementary experiments analyzing different aspects of PEF treatment in mixed microbial systems. Experiment 1: Bacterial viability and viable counts were measured across a $3 \times 3 \times 2 \times 2$ factorial design with treatment (Control, PEF1, PEF2), growth phase (Day 1, 3, 7), time post-treatment (2 h, 24 h), and culture type (monoculture vs. coculture) as factors. Due to the complexity of interactions, treatment effects were analyzed using one-way ANOVA at specific time points and conditions of biological interest, followed by Tukey's HSD *post hoc* test for pairwise comparisons. Experiment 2: Protein extraction efficiency was evaluated using one-way ANOVA to compare treatments (PEF1, PEF2, HPH) within each culture type (monoculture vs. coculture), with $n = 3$ biological replicates per group. Experiment 3: Antimicrobial activity of algal extracts was assessed qualitatively using agar well diffusion assays, with quantitative growth monitoring analyzed by treatment and culture type.

Statistical significance was set at $\alpha = 0.05$. Data are presented as mean ± standard deviation. Significant differences between treatments are indicated by asterisks ($*p < 0.05$) based on one-way ANOVA with *post hoc* testing.

Results

PEF processing of algae in the presence of bacteria

To evaluate the effects of PEF on algae and bacteria throughout their growth cycle, we applied PEF treatments on days 1, 3, and 7 of cocultivation, based on the results of our previous study.[18] These time points corresponded to different growth phases: day 1 (bacterial peak growth, algal lag phase), day 3 (algal exponential growth, bacterial stationary phase, and some gradually started to die), and day 7 (algal stationary phase, with viable bacterial concentration reduced by approximately 90% compared with day 1). For comparison, identical PEF treatment protocols were applied to monocultures of algae and bacteria grown under the same conditions.

The results revealed that PEF treatment exhibited consistent inactivation efficiency in *C. vulgaris*, showing no dependence on the growth phase (Fig. 1A–C). Across all treatment days, algae inactivation exceeded 95% for PEF 1 and over 99% for PEF 2 in the coculture experiments. It should be noted that algae grown without bacteria were not shown separately, as they showed equal responses to PEF.

In contrast, the bacterial responses to PEF varied dramatically depending on the growth phase and cultivation conditions. When *Delftia* sp. was cultured alone, day 1 bacteria showed remarkable resistance to immediate PEF inactivation, with a significant decrease in viability (below 70%) observed only 24 h post-treatment (Fig. 1D). By day 3, PEF treatment resulted in immediate and substantial bacterial inactivation regardless of energy input (Fig. 1E), indicating increased susceptibility as bacteria entered the starvation phase. Background mortality in untreated bacterial monocultures reached 90 ± 3% by day 7 (due to nutrient depletion), making it difficult to distinguish the specific contribution of PEF (Fig. 1E).

In the cocultures, bacterial responses differed markedly from those in the monocultures. On day 1, only the higher-energy treatment (PEF 2) achieved significant bacterial

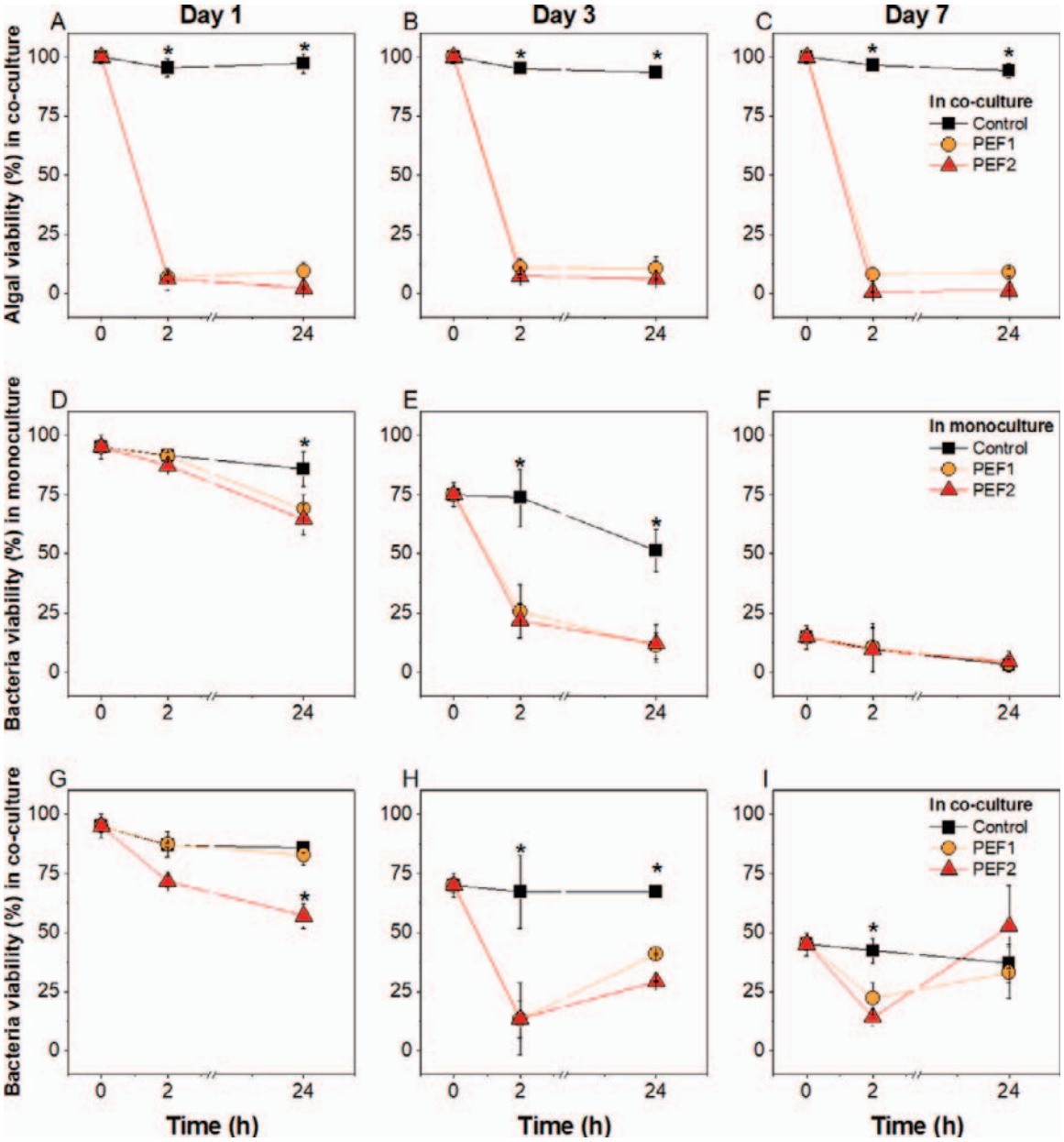

FIG. 1. PEF-induced changes in *Chlorella vulgaris* algae **(A, B, C)** and *Delftia* sp. bacteria viability when the bacteria were cultivated alone **(D, E, F)** or in coculture with algae **(G, H, I)**. PEF treatments were performed on days 1 **(A, D, G)**, 3 **(B, E, H)**, and 7 **(C, F, I)** of cultivation, and viability changes were determined 2 h and 24 h PEF post-treatment. Untreated samples were used as a control. PEF, pulsed electric field.

inactivation (50%) at 24 h post-treatment (Fig. 1G). Day 3 showed peak susceptibility with 70–80% viability reduction across both PEF settings at 2 h and 24 h post-treatment, respectively (Fig. 1H). On the final day (day 7), an unexpected trend emerged: bacteria in PEF-treated cocultures showed an initial viability reduction at 2 h post-treatment, followed by significant recovery at 24 h, with PEF 2-treated samples actually exhibiting higher bacterial viability (52%) than both untreated cocultures (35%) and PEF-treated bacterial monocultures (Fig. 1I).

To investigate this increase in bacterial viability by PEF, the viable bacterial concentrations in both monocultures and cocultures on day 7 were determined (Fig. 2). The results obtained supported previous data of bacterial viability (Fig. 1), showing that viable bacterial concentrations remained low in single-cultured samples across all treatments and time points (consistently below 6×10^6 cells/mL). In contrast, the cocultured samples showed increased viable bacterial concentrations following PEF treatment, particularly at 24 h post-treatment where concentrations reached approximately 2.3×10^7 cells/mL for PEF1 and 3.6×10^7 cells/mL for PEF2, compared with control levels of $\sim 1 \times 10^7$ cells/mL. Statistical analysis ($F_{2,6} = 7.25$, $p = 0.025$) confirmed significant treatment effects, with PEF2 treatment producing a significant increase in bacterial counts relative to controls ($p = 0.021$), representing an approximately 3.5-fold

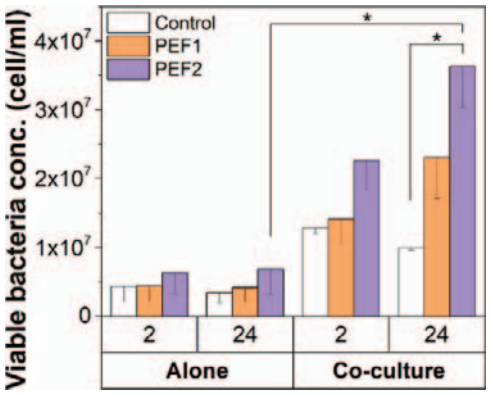

FIG. 2. Viable *Delftia* sp. bacteria count 2 h and 24 h post PEF treatment on day 7. Bacteria were cultured alone or in coculture with *C. vulgaris*.

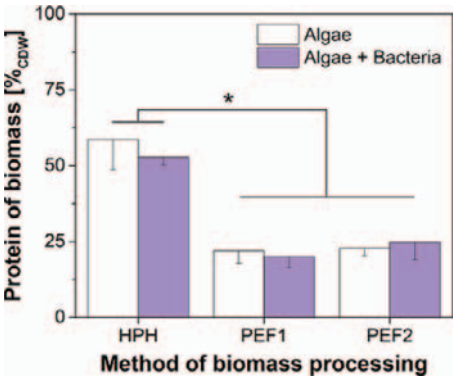

FIG. 3. HPH and PEF facilitated protein extraction from *C. vulgaris* biomass cultivated alone or in coculture with *Delftia* sp. bacteria.

increase. This proliferation effect was culture-dependent, as comparison between culture types revealed that PEF2-treated cocultures had significantly higher bacterial loads than corresponding monocultures at 24 h.

Antibacterial properties of C. vulgaris *extract*

To understand the mechanism underlying bacterial proliferation following PEF treatment of cocultures, we investigated whether compounds released from PEF-treated algae cells exhibited antimicrobial activity or could serve as growth substrates for bacteria. Two complementary approaches were employed: (1) assessment of the antibacterial activity of algal extract through agar diffusion assays and (2) evaluation of bacterial growth in PEF-processed algal extracts.

Agar diffusion tests with *Delftia* sp. showed no bacterial inactivation or formation of lysis zones around the wells containing algal extracts from either PEF or HPH treatments (Supplementary Fig. S1). To neglect the species-specific response to the algal extract, the same tests were conducted with *E. coli*, yielding similar results with no lysis zones (Supplementary Fig. S2). The absence of antibacterial activity in the algal extracts was confirmed by a second experiment in which microalgae extracts from PEF processing were used to culture bacteria. Spectrophotometric observations showed that optical density increased rapidly within the first day of cultivation and was similar to bacterial growth in fresh TAP medium containing acetic acid, an organic carbon source assimilated by both algae and bacteria (Supplementary Fig. S3).

Quantitative and qualitative properties of extracted proteins

To assess whether the presence of bacterial affects algal protein extraction and quality, we analyzed protein yields and profiles from PEF and HPH treatments of both monocultures and cocultures. Protein extraction yields showed no significant differences between algal monocultures and cocultures across different PEF treatments, with extraction efficiencies consistently reaching approximately 25%$_{CDW}$ (Fig. 3).

SDS-PAGE analysis revealed identical protein profiles in the PEF-treated samples, regardless of the presence of bacteria (Fig. 4). However, HPH extracts from the coculture samples produced a distinct protein band that was absent from the monoculture samples (Fig. 4, A-HPH and A + B-HPH traces, indicated by arrows). The molecular weight of this protein band, estimated from a calibration curve based on the molecular marker migration distances ($R^2 = 0.0992$), was approximately 27 kDa.

Discussion

PEF treatment effectively inactivated *C. vulgaris* cells even at low specific energy levels (4 J/mL; 40 kV/cm, 1 μs pulse), achieving >95% algal inactivation (Fig. 1A). This effectiveness is consistent with the >95% mortality reported by Krust et al.[17] (1.6 J/mL, 24 h after PEF), and aligns with the complete algal cell disruption (1.5 ± 0.7% recovery rate) demonstrated by Buchmann et al.[6] at comparable energy levels (3.8 J/mL). The effectiveness of this low-energy treatment likely results from CDIF-mediated cell death, as demonstrated by Krust et al.,[17] which explains the sustained algal inactivation observed at 24 h post-treatment, independent of additional energy input or growth phase. Cell inactivation resulted in consistent protein release patterns across different PEF conditions, achieving approximately 25%$_{CDW}$

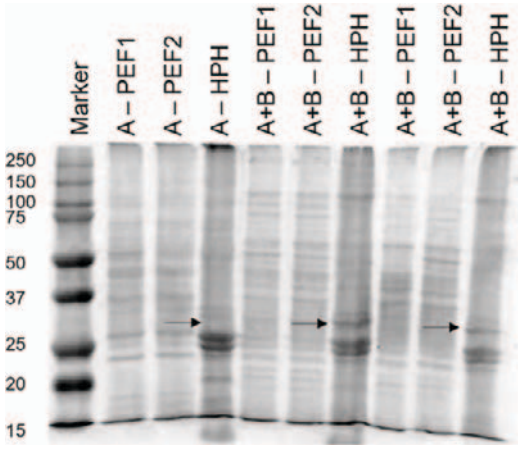

FIG. 4. Visualization of protein extracts obtained by HPH and PEF treatment by SDS-PAGE. Samples obtained from concentrated *C. vulgaris* biomass (5 g/L) cultivated alone **(A)** or cocultivated with bacteria **(A + B)**.

(Fig. 3). These values closely match the $22 \pm 2\%_{CDW}$ range reported by Scherer et al.[16] and Krust et al.[17] under comparable conditions. Notably, protein release remained consistent across different PEF conditions and was unaffected by the presence of *Delftia* sp. bacteria. SDS-PAGE analysis revealed that PEF maintained protein profiles consistent with previously reported molecular weight distributions for *C. vulgaris* (15–100 kDa range)[16] (Fig. 4). However, HPH treatment of cocultured samples produced an additional protein band ($\sim$27 kDa) that was not present in the algae-only extracts. Given that the applied HPH parameters disrupt bacterial cells, this band may originate from bacterial proteins, however, biochemical confirmation is required. Such proteins, if present, could compromise product quality for human consumption, as microbial contamination is recognized as the main cause of algae food safety issues.[30,31]

In contrast, PEF treatment of *Delftia* sp. demonstrated unexpected resistance to electrical disruption, with inactivation rates reaching only 25–90% even at high specific treatment energies (100 J/mL, PEF 2). This resilience is in contrast to typical responses reported for Gram-negative bacteria, where comparable energy levels (100–150 J/mL) generally achieve 2.0–3.5 $\log_{10}$ reductions, equivalent to 99–99.97% mortality.[19,32] In addition, the response of bacteria to PEF varied significantly with the growth phase, showing maximum sensitivity on day 3 in both mono- and coculture conditions. This peak coincided with the bacterial starvation phase, a period we previously identified when *Delftia* sp. depletes available carbon sources and enters the long-term stationary growth phase under these cultivation conditions.[18] While the growth phase dependency of PEF effectiveness has been documented in other bacterial species,[20,33,34] our findings present an interesting contrast to established patterns. Somolinos et al.[20] showed that exponential phase *E. coli* cells were consistently more PEF-sensitive than stationary phase cells, achieving approximately 5 $\log_{10}$ cycles of inactivation compared with less than 0.5 $\log_{10}$ cycles for stationary phase cells (50 pulses, 30 kV/cm). Similarly, Pothakamury et al.[34] reported that exponential-phase *E. coli* cells were more PEF sensitive than stationary- and lag-phase cells (2–4 pulses, 36 kV/cm, 2 μs). This conventional pattern, where stationary phase cells exhibit higher PEF resistance, has been linked to two main factors: the increased fragility of the cell envelope during exponential growth[35,36] and the larger surface area of actively growing cells, which enhances their vulnerability to electric field effects.[21] Understanding this distinctive response of *Delftia* sp. to PEF requires further analysis of starvation-induced cellular modifications, specifically examining membrane composition changes, stress response protein expression, and energy metabolism status during different growth phases.[20,33,37,38] Comparative studies between exponential and stationary phase cells would help determine whether this species exhibits unique vulnerabilities during nutrient limitation that affect PEF resistance.

From a bioprocessing perspective, the varying degrees of PEF susceptibility between algae and bacteria observed in our study present a significant advantage. Complete inactivation of algae, along with partial bacterial preservation, allows for selective cell disruption rather than bulk culture processing. This approach, particularly at lower energy levels, maintained bacterial viability (Fig. 1C) and possibly prevented introduction of additional protein bands observed with HPH treatment (Fig. 4). This pattern of selective cell inactivation is not unique to our system. Similar observations have been reported in other mixed microbial populations. Simonis et al.[39] achieved >3 $\log_{10}$ reduction of *Saccharomyces cerevisiae* (yeast) while reducing *Lactobacillus* (bacteria) populations by only 1–2 $\log_{10}$ (25 kV/cm, 100 μs) in acid whey.[40] Similarly, Rego et al.[40] demonstrated selective inactivation of predatory microorganisms (rotifers and ciliated protozoans) in *C. vulgaris* (microalgae) cultures (average 900 V/cm, 50 Hz, 65 μs) while maintaining algal viability. Such selectivity typically reflects fundamental biological differences between target organisms, including variations in cell wall structure, membrane composition, and cellular organization between prokaryotic and eukaryotic cells.

While algae showed high PEF susceptibility and bacteria demonstrated variable resistance, this differential response had unexpected consequences. *Delftia* sp. exhibited significant regrowth in the coculture with *C. vulgaris* by day 7, as shown in Figure 2. This bacterial growth is particularly notable given that day 7 cocultures have depleted available organic carbon sources from the original medium.[18] Under these carbon-limited conditions, the absence of antimicrobial activity in PEF-processed extracts, combined with observed bacterial growth in these extracts, suggests that algal cell disruption releases metabolizable compounds that can support bacterial growth when exogenous carbon sources are unavailable. Similar findings were reported by Ricós-Muñoz et al., who observed enhanced *Lactobacillus rhamnosus* growth in media supplemented with PEF-treated *C. vulgaris* and *Spirulina* extracts.[41] These findings highlight complex interactions in mixed-culture systems and emphasize the need for careful consideration in industrial applications, particularly in post-treatment processing steps.

Overall, these findings have important implications for the industrial application of PEF technology in mixed-culture systems. PEF treatment demonstrates valuable selectivity, effectively disrupting algal cells for protein extraction, while minimizing bacterial protein contamination at lower energy levels. This selectivity presents a distinct advantage for processing nonaxenic cultures, as protein extraction remains consistent despite the presence of bacteria. However, the observed bacterial proliferation during post-treatment processing indicates a risk that may extend beyond the immediate 24 h window. In typical protein recovery operations, biomass separation from supernatant is expected to retain bacteria in the pellet, but proliferating bacteria can produce secondary metabolites that may be toxic or compromise extract quality. Additionally, bacterial growth during extended processing may lead to protein and carbohydrate degradation, gas formation, spoilage, reduced shelf life, increased purification demands, and higher processing costs in downstream operations.[31,42–44] To maintain product quality and process viability over typical biorefinery processing timelines, manufacturers should consider incorporating rapid biomass separation, temperature control during processing, or antimicrobial preservation methods while preserving extract integrity.[31,45] These considerations apply to both PEF and HPH processing, though HPH may introduce bacterial protein

contamination in the extract, as suggested by the additional ~27 kDa protein band observed in our SDS-PAGE analysis (Fig. 4). Additional research focusing on the mechanisms of PEF's selective effects and bacterial metabolite production during post-treatment incubation would further enhance treatment protocols for mixed culture systems, ultimately improving industrial processing outcomes.

Conclusion

This study provides new insights into the complex, growth phase-dependent dynamics of PEF treatment in mixed microbial systems and its implications for biorefinery applications. We have shown that PEF consistently disrupted algal cells (>95% inactivation) regardless of the presence of bacteria, achieving protein extraction yields of approximately 25%$_{CDW}$. However, bacterial responses varied significantly with growth phase and culture conditions. Most notably, PEF treatment of cocultures led to unexpected bacterial proliferation (up to 3.5-fold increase) during post-treatment incubation. This resulted from algal cellular components functioning as metabolizable substrates rather than antimicrobial agents. From an applied perspective, our findings reveal both the opportunities and challenges for implementing PEF technology in industrial biorefinery settings. The selective nature of PEF-induced disruption offers advantages, preserving algal protein profiles while minimizing bacterial protein contamination compared to HPH. However, post-treatment bacterial proliferation necessitates a careful process design to ensure microbiological control throughout the extraction cascade.

Authors' Contributions

K.J.: Methodology, investigation, writing—original draft, and visualization. A.S.: Supervision and writing—review and editing. W.F.: Project administration and funding acquisition. C.G.: Conceptualization, methodology, and writing—original draft.

Author Disclosure Statement

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