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Hygienization of mixed animal by-product using Pulsed Electric Field: inactivation kinetics modeling and recovery of indicator bacteria

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Abstract

The hygienization of animal by-products (ABP) assisted by Pulsed Electric Field (PEF) was studied as a transfer of the innovative athermal pasteurization technology developed in food engineering to replace the conventional thermal hygienization process. The strains *Enterococcus faecalis* ATCC 19433 and *Escherichia coli* ATCC 25922 were selected as indicator bacteria. A systematical investigation was carried out concerning the inactivation kinetics of the indicator bacteria at different electric field strength (10, 15, 20 and 25 $kV \cdot cm^{-1}$), the kinetic modeling using coupled Weibull model, the effect of PEF energy input and the recovery of PEF-injured bacteria at 3 °C for 7 days. Results show that a PEF treatment at 25 $kV \cdot cm^{-1}$ for an effective time over 30 ms would be sufficient to achieve a 5-log10 reduction of *Ent. faecalis*. It complies with the EU regulation No. 142/2011 to validate an alternative hygienization technology other than thermal pasteurization. The Weibull model coupled with a secondary model proved efficient to estimate the survival kinetic curves of two indicator bacteria treated by PEF. The injured *E. coli* could not regenerate after PEF exposure while *Ent. faecalis* could get self-recovery. This preliminary work confirms that PEF was able to give the hygienization of ABP in terms of the bacterial reduction requirement of EU.

Keywords: Hygienization; Animal by-product; Pulsed Electric Field; indicator bacteria; Weibull model

1. Introduction

Animal by-products (ABP), as defined by the regulation of European Council No. 1069/2009, are the products of animal origin or those derived from animal transformation processes, which are not intended for human consumption [1]. ABP include slaughterhouse by-products (e.g. animal carcasses, blood and bones), waste from animal processing plants (e.g. wastewater sludge from slaughterhouses), animal slurry, etc. In 2014, all 28 member countries of the European Union (EU) generated 23.26 Mt of animal and mixed food waste. Besides, the production of animal faeces, urine and manure reached 12.83 Mt in the same year in EU [2]. These kinds of waste may harbor large varieties of infectious pathogens such as *Salmonella enterica*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, *Cryptosporidium, Giardia,* bovine spongiform encephalopathy agents and other hazardous virus [3–5]. The spread of antibiotic resistant microorganisms through the land application of contaminated animal slurry may also be a major concern [6]. Studies also show that improper transformation, management and disposal of ABP could bring about human and animal disease outbreaks threatening public health [7,8].

Anaerobic digestion (AD) is one of the promising technology that recovers the biomass energy from organic waste in biogas plants (BGP). It uses methanogenic bacteria, under controlled conditions, to transform the biodegradable organic matter into biogas. Different from other organic waste, the European Commission regulation EU No. 142/2011 requires that only those ABP classified as category 2 and 3 (e.g. manure, non-mineralized guano, digestive tract content and other less harmful animal residues) can serve as substrates for biogas production, on the condition that they are properly hygienized prior to AD process [9]. This mandatory hygienization of ABP is operated at

70 °C for at least 60 minutes without interruption (i.e. pasteurization), requiring the particle size being less than 12 mm. This thermal process represents around 6 - 19% of the primary energy produced in several European BGP [10], thus reducing the ecological gain as well as the financial interest of the BGP.

Pulsed Electric Field (PEF) is an innovative technology that has been widely investigated in the food processing industry for the purpose of the intensification of mass transfer effect (e.g. drying, extraction) and the non-thermal pasteurization. It inactivates the pathogenic microorganisms by provoking an irreversible electroporation effect on their cell membrane [11]. This novel technology proves to be a good alternative or complementary to the traditional thermal pasteurization thanks to its short treatment time in the order of milliseconds and high energy efficiency [12].

Other than thermal pasteurization, the European regulation EU No. 142/2011 entitles the EU member countries to the power of adopting alternative technology to hygienize ABP, provided that this novel approach can achieve a 5-log10 reduction of *Enterococcus faecalis* or *Salmonella Senftenberg* and a reduction of infectivity titer of thermos-resistant viruses by at least 3 log10.

Keles et al. (2010) [13] dealt with the application of PEF on the inactivation of *Salmonella* spp. in the waste activated sludge. However, according the EU regulations, the sewage sludge is not mandatory to be hygienized. In other studies, PEF served as a pretreatment method to enhance the methane production process of the biowaste (e.g. sewage sludge [14], pig liver [15] and pig slurry [16]). There has been a commercialized facility in the US specifically for this purpose [14]. For the time being, most of the research focus on PEF in the environmental engineering is confined to the methane potential enhancement and the dewaterbility of sewage sludge [17]. Meanwhile, similar

electro-technology, like pulsed power technique [18], pulse corona discharge [19] and electro-peroxone process [20], has been recently studied for the disinfection of drinking water and wastewater. Actually, little information is available about the use of PEF for a non-thermal hygienization of ABP under the EU requirement of the bacterial inactivation.

This study contributes to an initiative research into the feasibility of PEF as an alternative to the hygienization of ABP and discusses about its compliance with the European regulation mentioned above. The paper mainly deals with (1) the effect of electric field strength of PEF on the hygienization of ABP, characterized by 2 strains of indicator bacteria; (2) the kinetic modeling by using Weibull distribution model; (3) the effect of specific energy delivered by PEF on the pathogen inactivation and (4) the recovery of the PEF-injured indicator bacteria in ABP.

2. Material and methods

2.1 Mixed animal by-product

The mixed animal by-product was collected from a biogas plant LIGER (Locminé, France). It consisted of the local fishery waste and the swine slurry. The feedstock was at first mixed by industrial grinders and then the mixture was thermally pasteurized by BGP according to the European norm No. 142/2011 [9]. The product issued from the hygienization unit was collected using sterile material. The sample was divided into several sterile bottles under aseptic condition and stored at -18 °C for further study. The detailed physical, chemical and microbiological properties of this hygienized mixed ABP were presented in **Table 1**. It is worth noting that the total aerobic mesophilic count of this mixture was found at around 10^5 CFU·mL^{-1}, while no colony of either enterococci or *Escherichia coli* was identified by selective media.

2.2 Preparation of ABP suspension enumerated with indicator bacteria

Pure cultures of *Enterococcus faecalis* ATCC 19433 (Institut Pasteur, France) and *Escherichia coli* ATCC 25922 (Institut Pasteur, France) were chosen as indicator bacteria to characterize the treatment efficiency of PEF-assisted hygienization of ABP. *Ent. faecalis* ATCC 19433 (gram positive, "G+") was selected by the EU regulation as the indicator bacterium to validate an alternative technology of hygienization. It is also a suitable indicator for this study, neither too resistant or too fragile to the hygienization treatment [21]. The other strain, *E. coli* ATCC 25922 (Gram negative, "G-"), was selected because it has been widely acknowledged as a good indicator of faecal contamination in environmental studies [22].

The strains *Ent. faecalis* and *E. coli* were grown in the ordinary nutrient broth (CM0001, Oxoid, UK) at 37 ± 1 °C for 24 h and 20 h respectively to reach their stationary phase [23–25]. Then 1 mL broth of the incubated pure culture was diluted in a 9 mL Buffered Peptone Solution (CM0733, Oxoid, UK) tube. 0.5 mL of the diluted solution and 0.5 mL of the ABP sample were afterwards added into 9 mL sterile distilled water with its final electrical conductivity adjusted to 2000 $\mu S \cdot cm^{-1}$ and its pH at 6.8 - 7.2. The serial dilutions gave the enumerated suspension an initial microbial count of approximately 5×10^6 and 1×10^7 $CFU \cdot mL^{-1}$ for *Ent. faecalis* and *E. coli* respectively.

2.3 PEF treatment system

As illustrated by **Fig. 1**, the PEF treatment system was composed of a pulse generator (TGP 110, TTI Thurlby Thandar instrument, France), a high voltage power generator (SR2.5-P-600, Technix, France), a modulator (AHTPM2.5, Effitech, France), the electrical grounding and a treatment chamber. The system could provide mono-polar

square pulses with a maximum voltage of 2.5 kV, a pulse width between 1 - 100 μs and a repetition frequency of 24 - 240 Hz. An oscilloscope (OX8022-20 MHz, Metcix, France) was employed to regulate the pulse width and the repetition frequency. Electroporation cuvettes (1 mm Gap-90 μL, VWR, Belgium) were used as treatment chamber [26,27], connected to the PEF electric circuit. The inter-electrodes distance could obtain an electric field strength of 0 - 25 $kV \cdot cm^{-1}$ with the present PEF generator. To investigate the pure PEF treatment efficiency by avoiding ohmic heating, a mechanical air fan was applied to cool the chamber at real time.

2.4 PEF-assisted hygienization experiments

2.4.1 PEF treatment

The electroporation cuvettes were filled with 90 μL of the prepared suspension enumerated by one of the indicator bacteria and then connected to the PEF system. The inactivation kinetics of the target indicator bacterium at different electric field strength (10, 15, 20 and 25 $kV \cdot cm^{-1}$) were studied with the effective PEF treatment time (t_{PEF}) prolonged up to 30 ms. The t_{PEF} was calculated according to the **Eq. (1)**:

$$t_{PEF} = f \cdot t \cdot \tau \quad (1)$$

where f is the repetition frequency fixed at 40 Hz, t is the total duration of the treatment time for one trial (s) and τ is the pulse width fixed at 4 μs. With the aid of cooling system and the intermittent application of PEF through serial trains during one trial, the temperature of the suspension was kept below 45 °C to minimize the excessive thermal damage to the bacteria.

To investigate the effect of suspension medium on the pasteurization efficiency of PEF, the reductions of the two indicator bacteria in the ordinary nutrient broth (NB, adjusted to the same electrical conductivity as ABP suspension) were realized. The

samples in NB were treated by PEF at 10, 15, 20 and 25 kV·cm^{-1} for an effective PEF treatment time of 30 ms.

2.4.2 Viable counts

After each trial, the treated sample was rapidly chilled by iced water. The corresponding standard microbial analyses were performed in triplicate by spread plate method within 2 hours. According to Cunault et al. (2011) [28], *Ent. faecalis* were counted using Slanetz & Bartley medium (CM0377, Oxoid, UK) incubated at 37 °C for 48 h and *E. coli* were counted using Tryptone Bile X-Glucuronide (TBX) medium (CM0945, Oxoid, UK) incubated at 37 °C for 24 h.

2.4.3 Specific energy input

The specific energy delivered by PEF could be calculated from the following formula **Eq. (2)** [29]:

$$W_{PEF} = \int_0^{t_{PEF}} U \cdot I / v \, dt \qquad (2)$$

where W_{PEF} is the specific energy input of PEF (J·mL^{-1}), U is the applied voltage of the PEF treatment (V), t_{PEF} is the effective PEF treatment time (s), I is the electric current intensity (A) and v is the volume of the liquid treated (mL).

Three levels of energy input were chosen (300, 1000 and 3000 J·mL^{-1}). For each level, the survival ratios were studied at 10, 15, 20 and 25 kV·cm^{-1} for different treatment time to achieve the same specific energy input.

2.4.4 Recovery of injured indicator bacteria

Samples treated at 25 kV·cm^{-1} by PEF for 15 and 30 ms were investigated concerning the recovery of the PEF-injured indicator bacteria in ABP and compared to the untreated ones. This laboratory verification might reveal the treatment efficiency of PEF as a pasteurization method to which degree it inhibited the microbial activities of

the products. 25 µL of the treated ABP suspension was transferred into a sterile microplate (PS-96, Corning, US) filled with 225 µL nutrient broth. The broth was incubated at 3 °C and counted regularly for a period of 7 days to determine the evolution of the viable fraction of *Ent. faecalis* and *E. coli* respectively.

2.5 Mathematical model

2.5.1 Weibull distribution model

The inactivation curves of the indicators (survival fraction vs. t_{PEF}) were at first fitted to the Weibull distribution model [30], as shown in **Eq. (3)**:

$$\log_{10} N(t_{PEF}) / N_0 = - (t_{PEF} / \alpha)^{\beta} / 2.303 \quad (3)$$

where t_{PEF} is the effective PEF treatment time (µs), $N(t_{PEF})$ is the cell count of the indicator bacterium at instant t_{PEF} (CFU·mL^{-1}), N_0 is the initial cell count of the indicator bacterium (CFU·mL^{-1}), α is the Weibull scale parameter (µs) and β is the Weibull shape parameter (-).

2.5.2 Secondary model

A secondary model describing the relationship between the electric field strengths and the Weibull parameters (α and β) was proposed. It serves as a complementary model to the Weibull distribution in order to simulate the inactivation kinetics of indicator bacteria using one single equation with the electric field strength varied.

The scale parameter α was assumed following **Eq. (4)**, a function similar to the Arrhenius equation:

$$\ln 1 / \alpha = \ln A - B / E \quad (4)$$

where E is the electric field strength (kV·cm^{-1}), A (µs^{-1}) and B (kV·cm^{-1}) are the parameters of the secondary model depending on the nature of the target microorganism.

The shape parameter β, in reliability engineering, is independent of the external conditions [30]. Therefore, the values of β in this secondary model were fixed using the average of the β values extracted from the four inactivation curves of each indicator bacterium, denoted as $\bar{\beta}$.

By integrating the secondary model into the **Eq. (3)**, a coupled model was obtained as presented in **Eq. (5)**, used to predict the inactivation curves:

$$\log_{10} N(t_{PEF}) / N_0 = - \left[t_{PEF} \cdot A \cdot \exp\left(- B / E \right) \right]^{\bar{\beta}} / 2.303 \quad (5)$$

where t_{PEF} is the effective PEF treatment time (μs), $N(t_{PEF})$ is the cell count of the indicator bacterium at instant t_{PEF} (CFU·mL^{-1}), N_0 is the initial cell count of the indicator bacterium (CFU·mL^{-1}), A (μs^{-1}) and B (kV·cm^{-1}) are the parameters of the secondary model, E is the electric field strength (kV·cm^{-1}) and $\bar{\beta}$ is the average shape parameter of the Weibull model (-).

2.5.3 Calculation of the 5-D values

The parameter 5-D value is proposed, which corresponds to the treatment time needed to achieve a 5-log10 reduction of the targeted indicator microorganism, i.e. the bacterial hygienization of the product [23]. The 5-D values of each treatment were calculated according to the following **Eq. (6)**:

$$5 - D \text{ value} = \alpha \cdot (5 \times 2.303)^{1/\beta} \quad (6)$$

where α is the Weibull scale parameter (μs) and β is the Weibull shape parameter (-), both of which were obtained using the **Eq. (3)** and **Eq. (5)**.

2.5.4 Evaluation of modeling goodness

The goodness of the primary Weibull model and the coupled Weibull model was evaluated by the adjusted coefficient of determination (adjusted R^2), the sum of squared

errors (SSE) and the root mean squared errors (RMSE) [31]. The modeling with its goodness was processed by R studio (R Studio Inc., Massachusetts, US).

3. Results and discussion

3.1 Inactivation kinetics of indicator bacteria

3.1.1 Effect of electric field strength and effective treatment time

The inactivation kinetics of the two indicator bacteria in ABP realized by PEF at 10, 15, 20 and 25 kV·cm^{-1} were presented in **Fig. 2** with open symbols. *Ent. faecalis* achieved an inactivation of 0.70 ± 0.04, 2.43 ± 0.03, 3.58 ± 0.02 and 5.02 ± 0.03 log10 at 4 corresponding electric field while the decimal reductions for *E. coli* were of 2.15 ± 0.16, 2.74 ± 0.08, 3.47 ± 0.18 and 4.30 ± 0.01 log10 respectively. For comparison, the reductions of the two indicator bacteria in the ordinary nutrient broth (NB) provoked by PEF at 10, 15, 20 and 25 kV·cm^{-1} for t_{PEF} = 30 ms were investigated and presented in **Fig. 2** with closed symbols. Results show that for any electric field studied, we obtained a further reduction in NB as compared with ABP. The inactivation ratios were enhanced by 0.28 - 0.79 and 0.18 - 0.74 log10 for *Ent. faecalis* and *E. coli* respectively, when they were exposed to PEF in NB rather than in ABP. This means that the suspension liquid played an important role in the pathogen inactivation performance of PEF. Both two indicator bacteria in nutrient broth appeared to be more vulnerable to PEF treatment than in ABP. It implies a protective effect of ABP for the microorganisms to survive during the PEF exposure. This phenomenon could be explained by the presence of lipids, proteins and other complex chemical substances in ABP that protected the microorganisms from PEF stress [32].

Lower electric field (10 kV·cm^{-1}) had little effect on the inactivation of *Ent. faecalis* (0.5 log10) while *E. coli* were significantly inactivated (2.15 log10) at this level. For

both two bacteria studied, more microbial inactivation was observed when the field strength was increased. On one hand, *Ent. faecalis* was found more resistant to PEF during the first 5 ms than *E. coli*, considering that the survival curves of the latter dropped more rapidly. On the other hand, when the effective treatment time prolonged, particularly treated at 25 $kV \cdot cm^{-1}$, the strain *Ent. faecalis* became more vulnerable than *E. coli*: the survival curve of *E. coli* was stabilized at a reduction of around 4.30 log10 during the period of 20 - 30 ms but the curve of *Ent. faecalis* continued to drop off, exceeding a reduction of 5 log10.

Two indicator bacteria presented different behaviors with regard to the inactivation kinetics induced by PEF. *Ent. faecalis*, compared with *E. coli*, was more resistant during the first 5 ms at any field strength tested. This confirms the previous studies concluding that "G+" bacteria are generally more resistant to PEF than "G-" bacteria because of their different chemical composition of the bacterial wall and membrane [33,34]. However, with the treatment time extended, the former began to be much more vulnerable, achieving a 5-log10 reduction while *E. coli* reached only 4.3 log10 at 25 $kV \cdot cm^{-1}$. The mechanism related to this observation on a biological basis remains unclear. Wang et al. (2018) [11] reviewed that the protective mechanisms of a specific microorganism against PEF exposure might depend on various biochemical mechanisms, such as the reparation of cytoplasmic membrane, the changes of metabolic activities, the response of microbial oxidation stress and the glutathione-dependent biochemical defense. Moreover, García et al. (2006) [35] reported the capability of *E. coli* to repair the cell membrane damaged by PEF treatment through lipid synthesis during the exposure. This might be a possible explanation why *E. coli* had a high inactivation rate at first but gradually turned to be more PEF-resistant at last.

The microbial inactivation proved to be strongly correlated to the electric field strength and the treatment time as well. According to the EU norm No. 142/2011, a PEF treatment at 25 $kV \cdot cm^{-1}$ for over 30 ms would be sufficient to achieve the bacterial hygienization of the studied ABP (5-log10 reduction of *Ent. faecalis*). In fact, this achievement is attributed to the electroporation effect induced by PEF without the intervention of ohmic heating, which in industrial practice is preferred along with PEF to enhance the pasteurization efficiency [36]. Besides, there are studies showing that the coupling of the electrical and thermal effect has shown a synergetic effect that, in addition to the inactivation of vegetative bacteria, can reduce the bacterial spores, a resistant form that conventionally cannot be impacted by moderate PEF or thermal pasteurization at 70 °C [37]. Moreover, a previous study showed that damages could take place for the spores of *Bacillus pumilus* after 10 000 pulses of PEF (τ = 5 μs) at 7.5 $kV \cdot cm^{-1}$ [38].

In addition to the effect of pathogen inactivation, the EU hygienization condition (70 °C for 60 min) of ABP proved to be a pretreatment step that might influence the following biogas production kinetics in anaerobic digesters. Studies show that positive, null or negative effect could be obtained by this thermal pretreatment depending on the nature of feedstock [39–42]. Similarly, several studies concluded that the bio-methane potential of ABP could be enhanced by the pretreatment of PEF. They reported an increase of methane potential of 58% and 80% for pig manure [14,16] and 10% for pig liver [15]. This indicates the application of PEF in the field of biogas production is promising and in need of further studies concerning its joint effect on the overall processes, from pathogen inactivation to the biogas production, in the biogas plant.

3.1.2 Primary Weibull distribution model

The inactivation curves were fitted to the primary Weibull model as shown in **Eq. (3)**. The modeling results (values of parameters and modeling goodness) were resumed in **Table 2**. Almost all of the curves were fitted excellently (high R^2, low SSE and RMSE), except for those at 10 kV·cm^{-1} for both two indicators (adjusted R^2 = 0.777 - 0.781). The parameter α depended strongly on the electric field strength while for a given bacterium, β remained relatively constant. This corresponds to the previous studies where the scale parameter α was influenced by the external conditions (like pH, temperature, electric field) and the shape parameter β was expected to be constant for a certain microorganism or to be a weak function of the surrounding environment [43].

The corresponding 5-D values were given in **Table 2**. For both indicator bacteria the 5-D values were significantly reduced when the field strength was increased by every 5 kV·cm^{-1}. The 5-D value was estimated at 29.24 and 58.77 ms for the PEF-pasteurization of *Ent. faecalis* and *E. coli* at 25 kV·cm^{-1}.

3.1.3 Weibull model coupled with secondary model

The Weibull parameters of the four inactivation curves of each indicator bacterium were extracted in search of a secondary model describing their dependence on the electric field strength. The ln(1/α) was plotted against the reciprocal of the applied field strength (i.e. 1/E) for both indicator bacteria (not shown). A linear regression using **Eq. (4)** was performed and the fitting results were presented in **Table 3**. It was found that for both strains, the proposed linear model fitted the curves perfectly with the R^2 ranging between 0.988 - 0.991. As mentioned in the section 3.1.2, the variation of β was slight. Accordingly, the value of β in the secondary model ($\bar{\beta}$) was fixed at the average of the values extracted from the primary Weibull model for each strain respectively.

The obtained secondary model for two strains was then integrated into the primary Weibull model to exert one general model **Eq. (5)** that could predict the inactivation kinetics using one single equation for a given microorganism. The corresponding modeling results using this coupled model were shown in **Table 3** as well. The adjusted R^2 ranged from 0.734 - 0.976 and 0.763 - 0.991 for *Ent. faecalis* and *E. coli* respectively, indicating a rather good performance of the modeling. This statement was further strengthened by **Fig. 3** where the modeling results of the primary model and the coupled model were compared against the experimental values. The outcome of two modeling methods proved to be similar, which means that the coupled model gave a comparatively good prediction of the inactivation kinetics concerning the PEF-assisted hygienization of the ABP studied.

3.2 Effect of energy input of PEF

In this section, three iso-energy levels (300, 1000 and 3000 $J \cdot mL^{-1}$) were selected. The survival ratios of two indicator bacteria were studied for the same energy input but treated at different electric field strength, namely E = 10, 15, 20 and 25 $kV \cdot cm^{-1}$ (illustrated by **Fig. 4)**. Results show that with the same energy input of PEF, for both strains, the inactivation was strengthened when the electric field was increased. This implies that the electric field strength was one of the key parameters in the hygienization of ABP via PEF. However, in terms of *Ent. faecalis*, an exposure to 10 $kV \cdot cm^{-1}$ could only give rise to an inactivation of ~ 0.5 log10, regardless of the quantity of specific energy delivered. On the contrary, a reduction ranging from 0.73 to 2.15 log10 was achieved for *E. coli* when the specific energy varied from 300 to 3000 $J \cdot mL^{-1}$ at the same field strength (10 $kV \cdot cm^{-1}$). This phenomenon is coherent to a recent review stating that a lethal effect concerning the breakdown of microbial membrane could be

obtained at the electric field strength over 15 $kV \cdot cm^{-1}$ and significant microorganisms could survive when exposed to a moderate field strength between 10 - 19 $kV \cdot cm^{-1}$ [11].

It is worth noting that the energy input calculated in this paper was generally 10 times the energy input for a PEF pasteurization of *Lactobacillus plantarum* in a pH 4.5 phosphate buffers [44] and of *E. coli* in orange juice [45]. Grahl and Märkl (1996) reported that the energy input of PEF pasteurization of milk with 1.5% fat reached 600 $J \cdot mL^{-1}$ for a 6-log10 reduction of *E. coli* at 25 $kV \cdot cm^{-1}$ [46]. An inactivation of *E. coli* by 5 log10 in sodium phosphate buffer (pH 7) was obtained with an energy input over 1100 $J \cdot mL^{-1}$ at 33.3 $kV \cdot cm^{-1}$ [47]. Several explanation to this inconsistence of energy input may be attributed. Firstly, the microorganisms might be severely stressed in a low pH environment (fruit juice, low pH buffer) that made them more vulnerable to PEF exposure. Reversely, the pH neutral substrate (e.g. pH 7 phosphate buffer) and fatty products (e.g. ABP and milk) could give a protective effect to microorganisms from external injury. Secondly, the calculated energy input was nominal, which highly depended on the electrical efficiency of the voltage generator used.

3.3 Recovery and growth of PEF-injured indicator bacteria

Two indicator bacteria incubated in nutrient broth-ABP suspension at 3 °C for 7 days were examined for their recovery performance after the treatment by PEF at 25 $kV \cdot cm^{-1}$ for 15 and 30 ms. Results were shown in **Fig. 5** where two indicators presented different behaviors during the recovery time. The injured strain *E. coli* decreased to an undetectable level two days and one day after the exposure to PEF for 15 ms and 30 ms respectively. The untreated *E. coli* had the similar tendency, whose viable counts were reduced by 2.41 ± 0.04 log10 during the 7-day incubation. On the contrary, significant increase of *Ent. faecalis* could be observed for all treated and untreated samples several

days after the treatment. The population *Ent. faecalis* treated for 30 ms was seen regenerated one day later than the sample treated for 15 ms. On the 7th day after PEF injury, the treated enterococci achieved a recovery of 1.69 ± 0.23 and 1.82 ± 0.12 log10 respectively based on their initial counts. Slight growth (0.52 ± 0.14 log10) could also be observed for the untreated *Ent. faecalis* during the 7-day incubation.

The various recovery curves of two indicators revealed that the bacterial recovery depended strongly on their microbial nature. The inhibition of the untreated *E. coli* suggested the possible existence of microbial competition and environment changes (e.g. pH, chemical composition, nutrient acquirement) [48] that inhibited the regeneration of *E. coli* in ABP. In addition, although *E. coli* was found more resistant during a longer PEF treatment time, the injury induced by PEF proved to be persistent during the recovery time. It gave rise to a significant bacterial mortality (1.5 - 2 log10) two days after the treatment, more rapid than the intact samples (2.41 log10 in 7 d). When it comes to *Ent. faecalis*, its slow growth indicated the recovery and regeneration of the bacterium from the PEF stress. Considering the similar tendency of the intact samples, it might be concluded that the given PEF operational conditions (25 kV·cm^{-1}, 30 ms, 40 Hz) had limited effect on the inhibition of *Ent. faecalis*. A more stressful treatment may thus be necessary concerning this issue.

4. Conclusions

The PEF-assisted pasteurization at 25 kV·cm^{-1} for 30 ms could achieve the hygienization of ABP with a 5-log10 reduction of *Ent. faecalis*, conforming to the EU standard as an alternative hygienization technology for bacterial reduction. The coupled Weibull model could safely estimate the inactivation kinetics for two indicator bacteria. Besides, the study on the bacterial recovery indicated that the injured bacteria presented

different behavior of the self-recovery after the exposure to PEF. Further investigations should be focused on the optimization of PEF-assisted hygienization of ABP in terms of treatment mode (e.g. continuous system) and operational parameters (e.g. electric field strength, energy consumption). The joint effect of this innovative technology on both the pasteurization efficiency and the methane potential enhancement could also be a perspective of the research.

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Tables

Table 1

Characteristics of thermally hygienized animal by-product used as suspension media.

Parameters	mean ± s.d.[a]
Physicochemical parameters	
pH (-)	6.75[b]
Water content (%)	85.1 ± 0.1
TS (%)	14.9 ± 0.1
VS/TS (%)	94.5 ± 0.1
Electrical conductivity ($\mu S \cdot cm^{-1}$)	14 000[b]
Bio-chemical parameters	
COD ($g\ O_2 \cdot kg^{-1}$)	231 ± 1
TN ($g\ N \cdot kg^{-1}$)	6.02 ± 0.64
TP ($g\ P \cdot kg^{-1}$)	0.75 ± 0.01
Bacterial populations	
Total mesophilic count ($10^5\ CFU \cdot mL^{-1}$)	1.07 ± 0.09
Enterococcus spp. ($CFU \cdot mL^{-1}$)	N.D.[c]
Escherichia coli ($CFU \cdot mL^{-1}$)	N.D.[c]

[a] determined in triplicate except for pH and electrical conductivity.

[b] measured at 20 °C.

[c] "N.D." stands for "Not Detected".

Table 2

Primary Weibull distribution parameters, the corresponding curve fitting goodness of the PEF inactivation kinetics and the estimated 5-log10 reduction time (5-D) at different electric field strength for *Ent. faecalis* and *E. coli* (mean ± s.d.).

Electric field ($kV \cdot cm^{-1}$)	Individual Weibull Model					
	α values (μs)	β values (-)	adjusted R^2 (-)	SSE (-)	RMSE (-)	5-D values estimated (ms)
***Enterococcus faecalis* ATCC 19433**						
10	3 045 ± 160	0.2650 ± 0.0071	0.781 ± 0.148	0.055 ± 0.031	0.081 ± 0.024	31 400 ± 9 290
15	82.72 ± 23.82	0.2975 ± 0.0115	0.960 ± 0.031	0.129 ± 0.105	0.121 ± 0.054	301.3 ± 8.0
20	26.88 ± 6.081	0.3084 ± 0.0112	0.922 ± 0.007	0.753 ± 0.057	0.274 ± 0.010	73.73 ± 4.35
25	5.767 ± 0.735	0.2865 ± 0.0078	0.981 ± 0.014	0.388 ± 0.272	0.174 ± 0.065	29.24 ± 3.03
***Escherichia coli* ATCC 25922**						
10	15.47 ± 1.33	0.1775 ± 0.0035	0.777 ± 0.041	0.496 ± 0.122	0.248 ± 0.031	14 882 ± 2 785
15	1.187 ± 0.547	0.1763 ± 0.0053	0.890 ± 0.115	0.334 ± 0.379	0.231 ± 0.164	1 187 ± 72
20	0.1601 ± 0.0874	0.1749 ± 0.0073	0.936 ± 0.001	0.518 ± 0.007	0.227 ± 0.002	176.4 ± 4.7
25	0.0489 ± 0.0029	0.1746 ± 0.0011	0.966 ± 0.008	0.484 ± 0.116	0.192 ± 0.023	58.77 ± 1.50

Table 3

Parameters of the secondary model, the Weibull distribution model coupled with the secondary model and the corresponding curve fitting goodness at different electric field strength for *Ent. faecalis* and *E. coli* (mean ± s.d.).

Indicators	Secondary model			Weibull Model coupled with secondary model						
	A values (μs^{-1})	B values ($kV \cdot cm^{-1}$)	R^2 (-)	Electric field ($kV \cdot cm^{-1}$)	α values[a] (μs)	β values (-)	adjusted R^2 (-)	SSE (-)	RMSE (-)	5-D values estimated (ms)
***Enterococcus faecalis* ATCC 19433**										
	8.368	101.1	0.991	10	2927	0.2893	0.734 ± 0.095	0.074 ± 0.025	0.096 ± 0.017	13 639
				15	100.8	0.2893	0.902 ± 0.056	0.365 ± 0.227	0.208 ± 0.068	470
				20	18.70	0.2893	0.927 ± 0.005	0.282 ± 0.005	0.282 ± 0.005	87.15
				25	6.807	0.2893	0.976 ± 0.006	0.529 ± 0.111	0.209 ± 0.022	31.72
***Escherichia coli* ATCC 25922**										
	774.3	95.59	0.988	10	18.29	0.1758	0.763 ± 0.030	0.525 ± 0.100	0.256 ± 0.024	19 911
				15	0.7561	0.1758	0.891 ± 0.117	0.515 ± 0.548	0.292 ± 0.188	822.9
				20	0.1537	0.1758	0.981 ± 0.002	0.521 ± 0.012	0.228 ± 0.002	167.3
				25	0.0591	0.1758	0.991 ± 0.002	0.528 ± 0.130	0.201 ± 0.025	64.32

[a] The Weibull parameter α at different electric field strength was calculated by the secondary model.

Figures

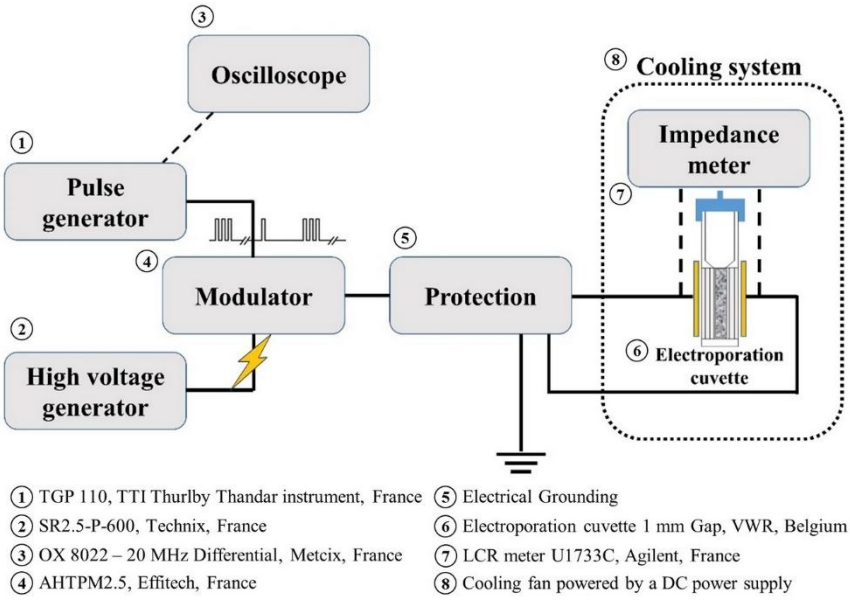


Fig. 1. Diagram of the experimental PEF treatment system.

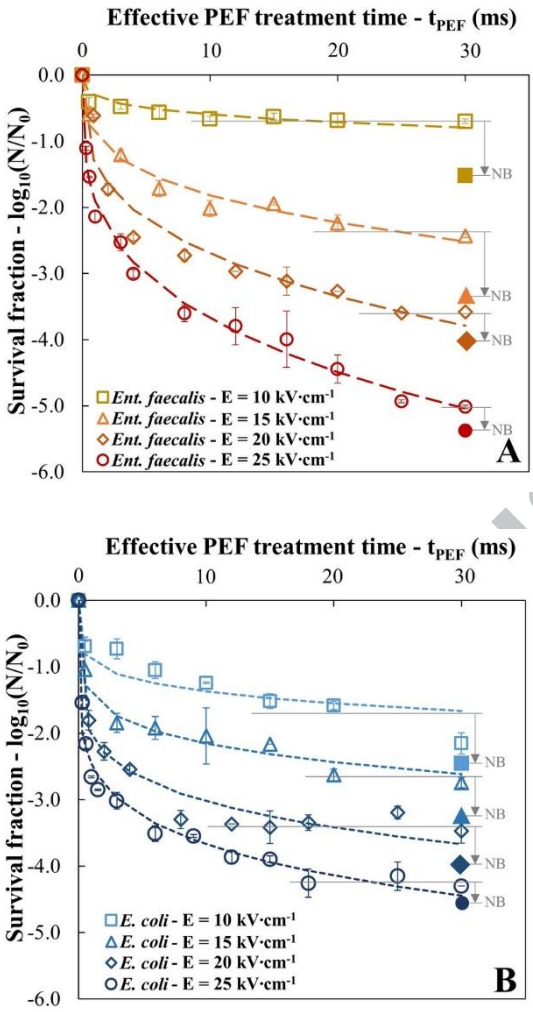


Fig. 2. PEF-assisted inactivation of indicator bacteria in animal by-products (ABP) as a function of effective PEF treatment time (t_{PEF}) using different electric field strength (E). A) *Ent. faecalis*; B) *E. coli*. Open symbols represent the experimentally observed survival fraction in ABP; error bars represent the standard deviations; dash curves represent the modeling results using the primary Weibull model; closed symbols represent the survival fraction of indicator bacteria treated at the corresponding electric field strength in the ordinary Nutrient Broth (NB).

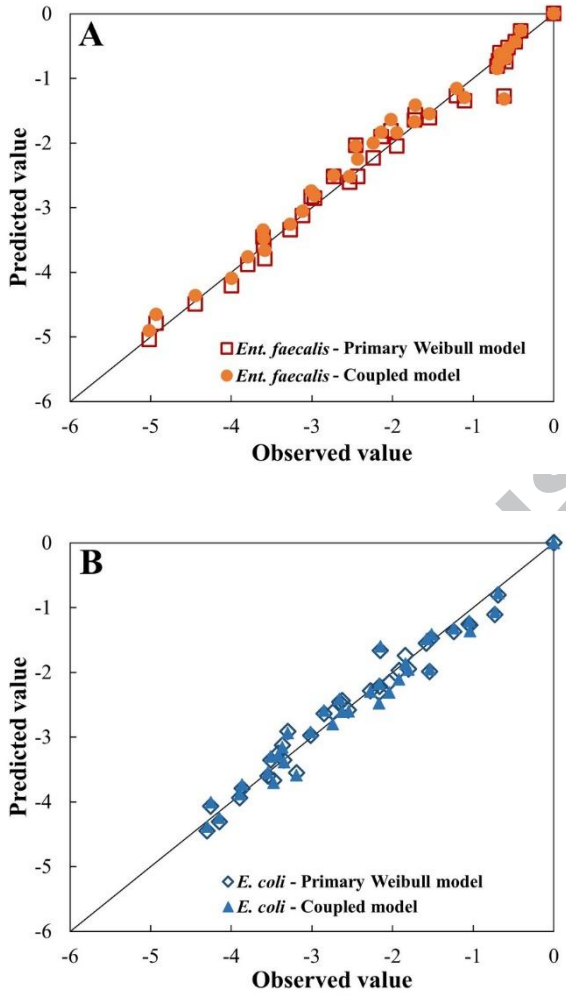


Fig. 3. Experimentally observed survival fraction versus predicted values calculated from the primary Weibull models and the Weibull model coupled with the secondary model for A) *Ent. faecalis* and B) *E. coli*.

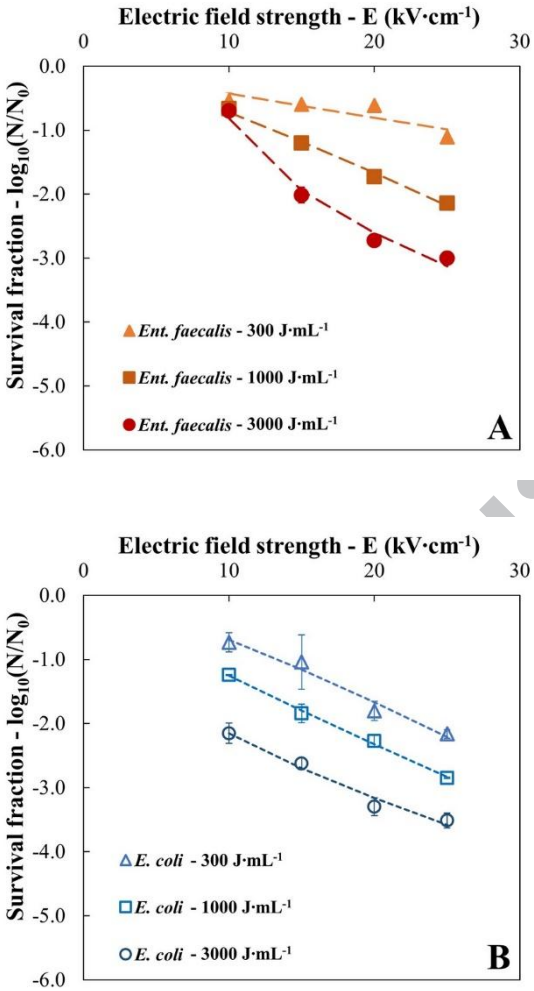


Fig. 4. Inactivation of A) *Ent. faecalis* and B) *E. coli* as a function of electric field strength at three levels of specific energy injected by PEF (dotted curves are the modeling results of Weibull model used as reference iso-energy curves, no physical meaning attributed).

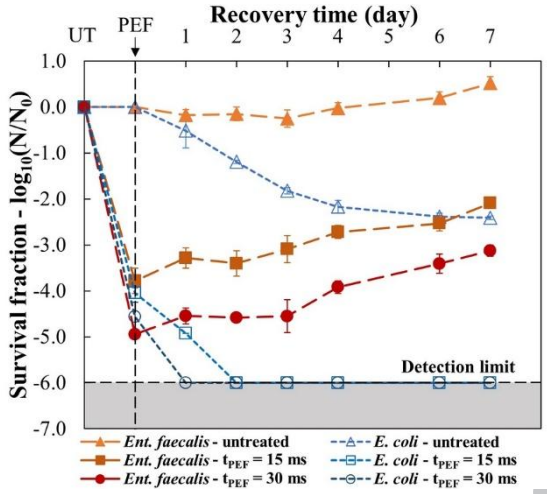
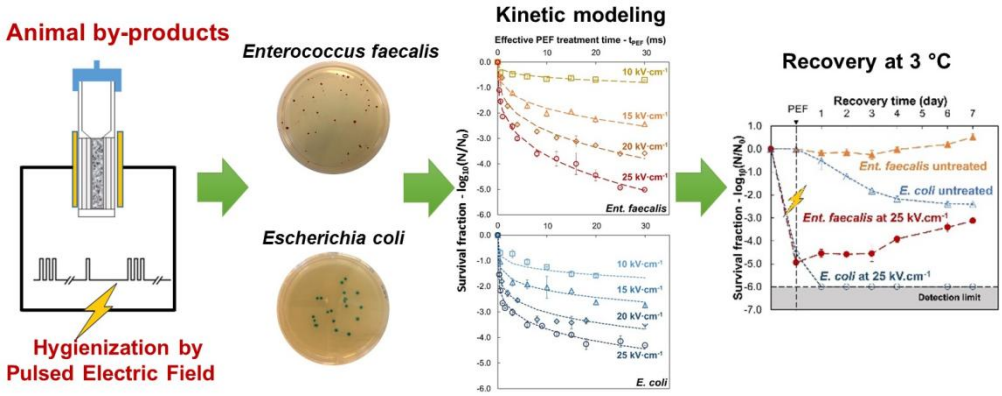


Fig. 5. Recovery and growth of untreated control and PEF-injured indicator bacteria (25 $kV \cdot cm^{-1}$ for t_{PEF} = 15 and 30 ms), incubated in nutrient broth at 3 °C for 7 days and counted by selective media. UT represents the untreated stage before PEF exposure.



Highlights

- Transfer of innovative food pasteurization technology to animal waste hygienization
- PEF can achieve the hygienization of animal by-product, conforming to EU regulation
- PEF treatment at 25 kV·cm^{-1} can give a 5-log10 reduction of *Ent. faecalis*
- Survival kinetics of indicators can be estimated by coupled Weibull model
- Recovery of PEF-injured indicator bacteria in ABP depends on their species