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Article

Induction of liquid bacterial and solid fungal cultures by conductive electrostimulation

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Abstract: This work seeks possible stimulative/inductive effects of conductive electrostimulation with alternating current on fungi and bacteria as a proof-of-principle. An iontophoresis device was used on solid fungal Petri dish cultures of six species belonging to four divisions versus identical controls to record differences of mycelial diameter as growth indicator over time. Six bacterial species of different shapes and Gram status were also tested in liquid cultures without or with (simultaneously or consecutively applied) mild heat pasteurization, incubated and then pour-cultured after successive dilutions. Enumeration of the colony forming units, versus proper controls indicated the effect. The fungi showed conditionally increased growth. The bacteria also showed conditional stimulative effects expressed as increased growth, in electrostimulated-only cultures; or as decrease of the detrimental effects of the pasteurization, if subjected to any combined regimen. No uniform set of parameters for either kind of stimulative results was identified, the window of susceptibility depending obviously on the organism; but also on the nutrient substrate. The method fits to green economy if developed and massively applied to the production of biopharmaceuticals, bacteriotherapeutics, metabolites and other bioproducts/ bioprocesses, the probiotics being an early candidate for commercialization.

Keywords: probiotics; bacteriotherapeutics; inductive electrostimulation; iontophoresis; culturomics; foodborne pathogens

1. Introduction

The concept of inducing microbial growth by electromagnetic-EM modalities is well warranted in food industry, for microbial procession of edible products, especially liquids (beverages included) [1,2], in environmental microbiology, for bioremediation [3], and in biotechnology, for increased production parameters of different amenities of microbial bioorigin [4]. Still, it is nascent, even as a concept, in medical microbiology, despite well-substantiated promise [5], as the scope and intensity of possible, diverse interactions are difficult to assess and predict [6]. Its importance in drug industry, sensu lato, for Biopharmaceuticals (any kind of enzymes, bioregulators, co-factors produced in living cells) [7] is self-evident, although underappreciated to date. But in an era of increased interest in the regulatory and

therapeutic role of live microbiota preparations, as in probiotics [8,9] and in microbiotherapeutics (such as, but not limited to, bacteriotherapy in cancer [10]) the field seems of exceedingly promising economic importance, and with more direct applications than the suppressive, decontaminant or therapeutic antimicrobial EM amenities, which capitalize on different aspects of the Bioelectric Effect [11,12].

The explosive development of the probiotics and associated sectors and subsectors, such as the prebiotics [13,14], the nutribiotics and the pharmabiotics [15] has created great expectations in the fields of Medicine and Public Health as they may be used proactively, for enhancing health, and reactively, by assisting in the therapy of disease [16–18]. Fulfilling such high hopes is conditional to the progress achieved in Genomics [19] and in Synthetic Biology [20], especially within the microbiomics and metabolomics/interactomics contexts [21–23]. The sum of the three intertwined, or rather convoluted areas, may be considered something more than an improvement in economics [18] or a passing fashion in terms of consumption sprees [8]. The effect of an optimized diet improves health and welfare by providing more necessary nutrients and energy with lower consumption of food. It also results in a more robust immune system; all these depend to some degree on the different appendage microbiomes, but mainly on the one of the Gastrointestinal Tract—GIT which affects the Gut-Brain Axis—GBA [13,18,24]. The protective dimension as set may be extended further or expanded to include therapeutic interventions through pharmabiotics [15] while nutrigenomics is expected to evolve so as to include not only the genome of the host, but the hologenome, understood as the sum of the genomes of the constituents of the holobiont [25].

On the other hand, all possibilities to magnify benignly and enhance the intended effect of probiotics/pharmabiotics on-target, or even the likelihood to achieve similar effect by treating parts of the native appendage microbiome (s) [6], seem also promising in terms of cost-effectiveness of preventive and protective treatment. This manuscript reports pilot, proof-of-principle research and its objective is to explore the possibility to use electrostimulation-ES (understood as the use of electric modalities to induce different parameters of microbial growth) [26,27], and actually to turn electricity into a prebiotic for culturable microorganisms. It focuses on fungal solid and bacterial liquid cultures. The panel of the tested microorganisms was selected in order to test ubiquitous microorganisms with pathogenic potential within the One Health, expanded concept [28], but also with some beneficial prospective application. This panel consists of microorganisms of health the economic interest, including human and plant pathogens of environmental occurrence or inhalation/ingestion uptake. This is due to the priority funding of pathogens, regarding future in-depth, basic research; also their diversity, which allows testing of highly different organisms, and the ubiquity of strains and know-how. The expected results could be used initially in better planning the use of therapeutic electroceuticals and in enhancing production of biotechnologically relevant microorganisms and probiotics, irrespective of the current inclusion or not of the tested organisms in any of these categories [29]. For example, current probiotics are mostly bacterial [30], but the fungal arm of the probiotics might gain in importance [31].

2. Materials and methods

The methodology used in this study has been detailed previously for both mycelial fungi [26] and bacteria [27]. Briefly, however, the experiments were as follows.

2.1. Equipment

The electrostimulation was implemented in all experiments by the portable Mio-Ionotens® (I-Tech Medical Division, Martellago Italy) instrument, used either plugged in standard electric grid interfaces or as battery-powered to generate Transcutaneous Electrical Nerve Stimulation—TENS [32] and iontophoresis currents [33]. It features two independent channels, so as to run two different programs simultaneously; each channel may treat up to two targets by the use of the provided splitter cabling, thus dropping the intensity output to half the nominal value.

2.2. Cultures of microorganisms

2.2.1. Bacteria

Strains of six common Gram-positive and Gram-negative bacteria were obtained from National Collection of Type Cultures—NCTC. Pure cultures were prepared by inoculating 10ml Tryptone Soy Broth -TSB tubes with 1ml of previously prepared stock culture. Incubation was according to microbial species: *Escherichia_coli* NCTC 9001 was incubated at 37 ℃ for 24 h, *Salmonella enterica* subsp. *enterica* serovar *typhimurium* NCTC 12023 (thereafter called *Salmonella typhimurium*) at 37 ℃ for 48 h, *Campylobacter jejuni* NCTC 11322 at 37 ℃ for 48 h under microaerophilic conditions (by addition of 1 mL of sterile paraffin), *Staphylococcus aureus* NCTC 6571 at 37 ℃ for 48 h, *Bacillus cereus* NCTC 7464 at 37 ℃ for 48 h and *Clostridium perfringens* NCTC 13170 at 37 ℃ for 48 h. All these bacteria are conditionally pathogenic and may be implicated in foodborne disease, food spoilage but also in other concerns of public health; thus they constitute possible biosecurity issues [34–37]. Some of them have a potential as probiotics: Strains of *E. coli* may be considered for probiotics use [38], *B. cereus* conditionally so [39], and *Clostridium* sp only after testing and in special occasions [40]. The genera *Salmonella*, *Clostridium*, *Escherichia* are promising bacteriotherapeutic factors for cancer treatment [10], persistent strains of *S. aureus* alleviated Experimental Autoimmune encephalomyelitis—EAE, an animal model of Multiple Sclerosis—MS [41] while engineered *E. coli* strains may assist in bioremediation and circular economy by degrading plastics [42].

2.2.2. Fungi

The tested fungi were provided by the Hellenic Collection of Pathogenic Fungi, University of Athens—UOA/HCPF. These were: *Aspergillus niger* UOA/HCPF 10603C, *Fusarium oxysporum* UOA/HCPF 14172, *Pythium ultimum* Benaki Phytopathological Institute Collection—BPIC 1229, *Rhizoctonia solani* BPIC 2529, *Mucor* sp UOA/HCPF 11596, *Candida parapsilosis* American Type Culture Collection—ATCC 22019. The mycelial species were selected as cross-kingdom

pathogens (human and plant pathogens) [43,44], with the exception of the *P. ultimum* which has no human infectivity recorded to date, but is related to the conditionally anthropopathogenic species *P. insidiosum* [45]. The other four show some, more or less prominent, human pathogenicity [46–49]. The only yeast species tested, *C. parapsilosis*, is anthropopathogenic, without known plant hosts [50]. None of the tested fungal species is a recognized fungal probiotic agent, although *C. parapsilosis* belongs to the genus *Candida*, which includes a number of recognized probiotic agents, i.e. *C. tropicalis* [31].

2.3. Treatment of microorganisms

2.3.1. Electrostimulation of liquid bacterial cultures

The ES exposure of bacteria was a single boost treatment upon inoculation by Alternating Current—AC. The electrodes of the mio-IONOTENS device were inserted aseptically in the culture vessels (glass test tubes), each containing 5 ml fresh Tryptone Soy Broth—TSB (Neogen, USA) inoculated with the bacterium of choice (**Figure 1**). The 1st experimental setup (cultures treated by ES only) referred to four settings of ES parameters, produced by the combination of two intensity values, 1 mA and 10 mA (low and high intensity), with two frequency values, 2 Hz and 800 Hz (low and high frequency). Exposure for each combination was for 1 min, 10 min and 30 min (3 batches), resulting in a total of 12 electrostimulation-only treatments per strain (batches (times) ES combinations).

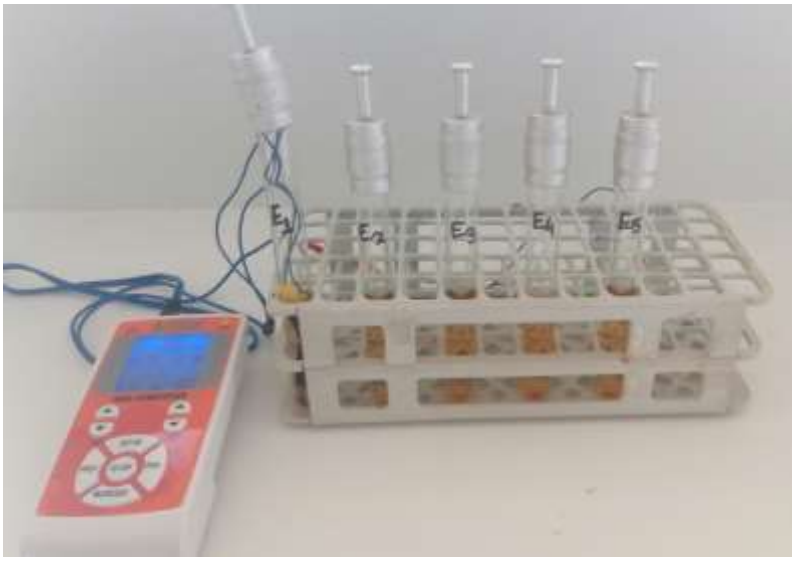

Figure 1. The ES format used for the treatment of bacterial liquid cultures.
The output electrodes are immersed sequentially in each one of the sample culture tubes. To achieve constant delay between the treatment sessions of successive samples, only one output channel is used, as obvious by the cables being attached to the left port of the device.

Identical ES treatment was combined with a standard thermal pasteurization step, using a LabTech water bath (Tecotec, Vietnam) set at 65 °C to test possible antagonism or synergy when combining ES with minimal thermal processing; such interactions are important for food industry and public health procedures. Two combinations of ES-pasteurization were tried, the *consecutive*, where the pasteurization step was implemented asynchronously, after the electrostimulation

treatment (2nd experimental setup), and the *simultaneous*, where ES and pasteurization were implemented synchronously (3rd experimental setup). Given that each setup, as mentioned above, included 12 tubes per strain, the three setups 36 tubes per strain, giving a total of 216 tubes for all six strains; this number discouraged any notion of repetitions within the framework of a pilot study.

2.3.2. Electrostimulation of solid fungal cultures

For the solid fungal cultures, the treatment duration was uniform; 15min. The assay cultures were produced by collecting spores from the mycelia of starter cultures or loopfuls of the yeast *C. parapsilosis* ATCC 22019 by sterile, disposable 1-μl inoculating loops next to Bunsen burner and placed by puncture, under aseptic conditions, to the geometrical center of Petri dishes containing 20 ml Sabouraud Dextrose Agar-SDA or Malt Extract Agar-MEA [51], and previously drilled aseptically to allow subsequent insertion of the pin electrodes (**Figure 2**). Two exposure patterns were tried: (i) Single boost treatment upon inoculation, conceptually identical to the exposure of the bacterial cultures; and (ii) regularly repeated treatment of daily exposure.

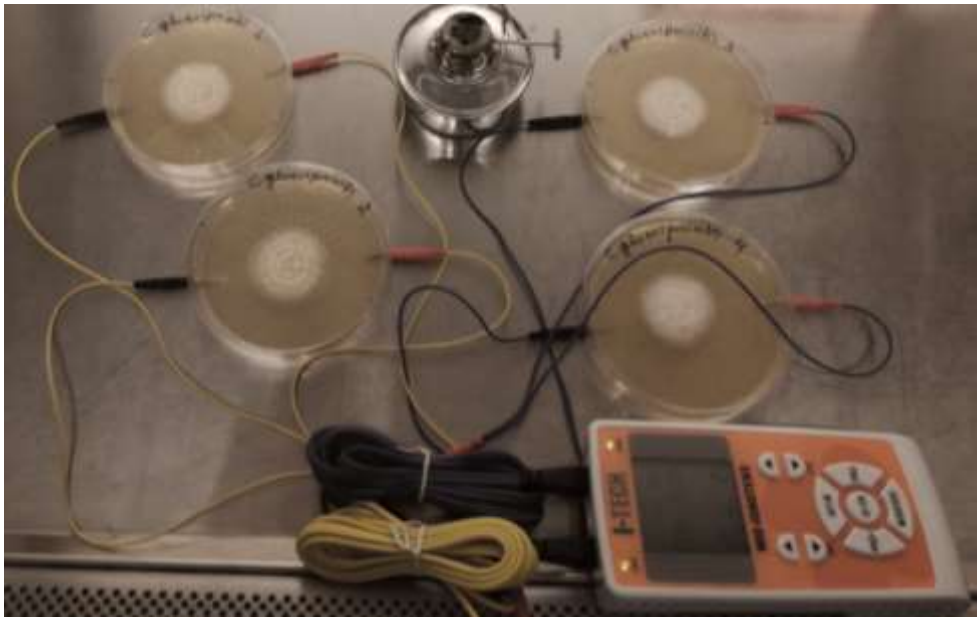

Figure 2. Indicative ES format for the treatment of fungal solid cultures.
The capacity for simultaneously treating four dishes refers to one quadruplicate treatment, as in here with Colletotrichum gloeosporioides. The setup is identical to the one used for the *F. oxysporum*. Two different ES settings may be applied concurrently, allowing two different duplicate treatment regimens; one per current output channel (identifiable by the color of the cable bunches projecting from the two ports of the device).

The drilling was performed by a microbiological steel needle of 0.2 cm diameter, heated in the blue flame of the Bunsen burner and melt-piercing the side-wall of the dish in two opposite spots, i.e. at an angular distance of 180°. Drilled dishes were stacked near the Bunsen burner to preserve aseptic conditions until inoculation and treatment. The pin electrodes were extracted after treatment and the dishes sealed by moisture-resistant, transparent, air-permeable, stretch self sealing film and incubated at room temperature, placed off direct sunlight.

The *Aspergillus niger* UOA/HCPF 10603C, *Pythium ultimum* BPIC 1229, *Rhizoctonia solani* BPIC 2529, *Candida parapsilosis* ATCC 22019 were tested in single experiments, exploiting fully the capability of the device to treat simultaneously two pairs of petri dishes (one dish treated daily, the other only once). Each pair was exposed to different intensity settings: 0.5 mA and 1 mA, and the quartet was accompanied by a single negative control dish, inoculated but untreated; the optimization of the controls has been described previously in detail [26]. The frequency of the current was set to the absolute minimum of the device (wave frequency: 1 Hz; wave impulse width: 250 μsec), to create as much as possible effects expected by Direct Current-DC.

A qualitative indicator of the ES of solid fungal cultures has been established previously [26], where multiple identical simultaneous treatments compared to a pair of identical controls allowed basic error and confidence analysis. Briefly, SDA dishes puncture-inoculated with *Fusarium oxysporum* UOA/HCPF 14172 were treated identically in quadruple, as in the ES setup shown above (**Figure 2**), and co-incubated with identically inoculated but unexposed pairs of control dishes under two exposure patterns, as above. Conditions were selected so as to test a single intensity setting (1 mA) in the lowest frequency settings allowed by the iontophoresis instrument, as detailed above.

2.4. Microbial growth assessment

To introduce some common ground between two entirely different kingdoms and experimental procedures, i.e. liquid bacterial cultures and solid fungal ones, the metric selected was the percent (%) increase of growth in treated sample (s) relative to the respective controls at selected time-points: One in bacteria, several in fungi.

2.4.1. Enumeration of bacterial populations

The bacterial samples were treated once, upon inoculation, and were quantified once (end-point assessment). This was after the end of the respective incubation in a dry heat incubator set at 37 ℃ and of duration dependent on species, as detailed in Subsection 2.2.1. The end-point assessment was implemented by viable counts of colonies produced by the pour plate method, by transferring 1 ml of the liquid culture and of 4 successive decimal dilutions in clean Petri dishes, where warm molten substrate was poured, mixed and left to solidify and then incubated at 37 ℃ in a dry heat incubator as described elsewhere [27]. The mean bacterial population (number of colonies) of three successive decimal dilutions was counted in accordance with the standard criteria of microbiological enumeration methods of the American Society of Testing and Materials—ASTM (1998) and expressed in colony forming units per ml of liquid inoculum (cfu/ml). Selective solid substrates were used for each bacterial species (all purchased from NEOGEN, USA), to expedite their growth for a relatively fast reading, and in line with standard enumeration practices for bacterial load, but also deleted any contaminating microbial species, thus ensuring a much more representative and reliable viable counts.

E. coli was inoculated into TBX agar and incubated at 37 ℃ for 24 h, *S. typhimurium* was inoculated into XLD agar and incubated at 37 ℃ for 24 h, *Campylobacter jejuni* was inoculated into Campylobacter Selective agar with

Campylobacter selective supplement and incubated at 37 ℃ for 48 h under microaerophillic conditions (in 5% CO_2 incubator), *Staphylococcus aureus* was inoculated into Baird Parker agar with egg yolk tellurite and incubated at 37 ℃ for 48 h, *Bacillus cereus* was inoculated into Bacillus cereus agar with egg yolk and polymix B supplement and incubated at 37 ℃ for 24–48 h, *Clostridium perfringens* was inoculated into TSC agar and incubated at 37 ℃ for 48 h in anaerobic jar with Anaerogen (OXOID, UK).

2.4.2. Assessment of growth of fungal colonies

The assessment of the fungal growth was performed repetitively by measuring the diameter of the fungal colony/mycelium daily or by two-day intervals, as previously reported [26]. Briefly, the said diameter was measured for all dishes—irrespective of treatment—before the electrostimulation session of the day, by marking the edges of the longer colony diameter with fine marker to enhance accuracy and then counting the distance with a commercially available desk ruler, without inverting the dish, to avoid dispersion of fungal spores or cells that would produce secondary growth foci. Measurements were plotted to time-growth curves. In the quadruple *F. oxysporum* UOA/HCPF 14172 experiment, averages were calculated for the four identically treated dishes and for the control couples and plotted on time-growth curves. Independent *t*-test was used to determine statistical significance of observed differences between control and treatment averages in the daily measurements. *P* values of < 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of electrostimulation on bacteria

The maximum inductive effect for the six bacteria is expressed as per cent (%) increase of the cfu/ml metric, compared to respective controls and as a function of ES exposure duration and instrument settings (**Table 1).**

Table 1. Electrostimulation of liquid bacterial cultures.

Bacterium	Strain ID (NCTC #)	Gram (+/−)	Exposure (min)	ES settings	Pasteurization (Consecutive/Simultaneous/None)	Max Growth increase (%)
B. cereus	7464	+	1	2 Hz, 1 mA	simultaneous	1000
C. jejuni	11322	−	1	800 Hz, 10 mA	simultaneous	339
C. perfringens	13170	+	10	800 Hz, 10 mA	No	104
E. coli	9001	−	10	2 Hz, 1 mA	No	158
S. typhimurium	12023	−	30	800 Hz, 1 mA	No	145
S. aureus	6571	+	1	2 Hz, 1 mA	No	110

The inductive effect of electrostimulation on six (6) bacterial strains (identified by Strain ID# and species name) as a function of Gram status, AC intensity and frequency, exposure duration per treatment session and simultaneous or consecutive Pasteurization or lack of it. The inductive effect is expressed in the last column as per cent (%) increase of the cfu/ml viable count metric, compared to the concurrent respective unexposed controls. A species might demonstrate induction in more than one sets of conditions, as with *S. aureus*; only the maximum induction is presented herein.

In detail (**Figure 3)**, the NCTC 9001 of *E. coli* was induced by simple ES exposure of 10 min at 1 mA irrespective of the used frequency, 800 Hz or 2 Hz. The NCTC 12023 of *Salmonella typhimurium* was induced by simple ES exposure of 30 min at 1 mA and 800 Hz, similarly to the *E. coli* NCTC 9001.

The NCTC 7464 of *B. cereus* showed a considerably different pattern: Exposure to ES for 1 min with simultaneous pasteurization showed clearly inductive results under all four ES settings. The same happened when subjected to pasteurization after the ES challenge in all ES settings but for the fourth combination, with high ES settings (800 Hz, 10 mA). The treated strain NCTC 13170 of *Clostridium perfringens* differed greatly from the treated strain NCTC 7464 of *B. cereus*; it was induced by simple ES exposure of 10min at the high extreme combined ES settings, 10 mA and 800 Hz and of 30 min exposure at 2 Hz, 10 mA under simultaneous pasteurization.

The NCTC 6571 of *S. aureus* was induced by stand-alone ES treatment of 1 min at 1 mA irrespectively of the used frequency, 800 Hz or 2 Hz; similarly to the respective of *E. coli* NCTC 9001, although at much briefer exposure time. When ES was followed by pasteurization, the extreme time of exposure and settings proved inductive for *S. aureus* NCTC 6571 (short time and combined low ES settings: 1 min 2 Hz, 1 mA; long time and combined high ES settings: 30 min 800 Hz, 10 mA), while all ES settings proved inductive for 1 min exposure with simultaneous pasteurization. The NCTC 11322 of *C. jejuni* showed induction when exposed to combined pasteurization (both synchronous and asynchronous) and ES treatments of 1min and combined high settings.

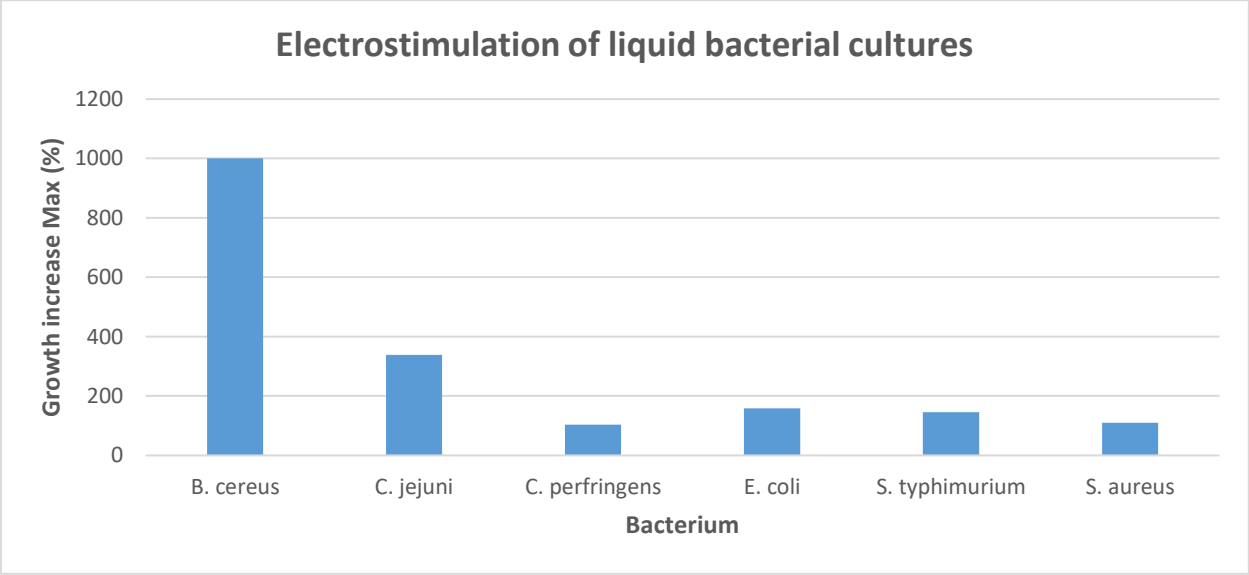

Figure 3. The inductive effect of ES on the tested bacteria, according to data of **Table 1**.

The inductive effect is expressed as per cent (%) increase of the cfu/ml viable count metric in pour cultures of samples and concurrently processed respective unexposed controls. Different species show induction under different ES settings (see **Table 1**). A species might demonstrate induction in more than one sets of conditions; only the maximum induction is considered herein.

3.2. Effect of electrostimulation on fungi

For the fungal strains of **Table 2,** the maximum inductive effect was expressed as per cent (%) increase of colony diameter (or of the respective average where

applicable) compared to the control (s) and is reported in terms of exposure pattern, current intensity, substrate and day of maximization**.**

In detail (**Figure 4)**, stimulative ES effect of kinetic nature was detectable in multiple fungi grown in SDA: The BPIC 2529 of *Rhizoctonia solani* was stimulated by 1 mA under exposure pattern (i), as defined in Subsection 2.3.2 above, while all other settings produced no appreciable change in growth. The BPIC 1229 of *P. ultimum* was stimulated by relatively low accumulative charges: By 0.5 mA under both exposure patterns and by 1 mA under exposure pattern (i). The ATCC 22019 of *C. parapsilosis* was stimulated by 0.5 mA under both exposure patterns. The UOA/HCPF 10603C of *A. niger,* was stimulated in SDA, by 0.5 mA under exposure pattern (ii) and in MEA, by 1 mA under both exposure patterns.

Daily conductive treatment of the *F. oxysporum* strain UOA/HCPF 14172 in SDA dishes showed, as expected, suppressive effect, demonstrated by slower growth than the respective untreated controls. But if treated only once, upon inoculation, from day 5 till day 8 the treated dishes displayed increased growth compared to controls, and this continued with levels of statistical significance ($P < 0.05$, Controls versus Samples average). In later days the controls caught up in terms of growth and it seems that the plateau was identical for once-treated and untreated control dishes, substantiating Kinetic difference (discussed in the Discussion section).

Table 2. Electrostimulation of solid fungal cultures.

Fungus	**Substrate**	**Strain ID**	**ES intensity(mA)**	**Exposure pattern**	**T_{IDE}**	**IDE (%)**
R. solani	SDA	BPIC 2529	2	Once	7	11.6
P. ultimum	“	BPIC 1229	1	Once	7	33
C. parapsilosis	“	ATCC 22019	1	Once	4, 5	20
F. oxysporum	“	UOA/HCPF 14172	1	Once	5	6
A. niger	“	UOA/HCPF 10603C	2	Daily	6	17
A. niger	MEA	UOA/HCPF 10603C	2	Daily/Once	3	33
Mucor sp	MEA	UOA/HCPF 11596	1	Daily	10	27.5

The effect of ES on solid fungal cultures of six strains identified by species names and Strain ID# as a function of solid substrate, AC intensity, and exposure pattern. One strain (Aspergillus niger) is used twice, in both substrates, thus totaling seven test cultures. The day after inoculation during which the treated dish of each strain showed the maximum positive difference in growth to the respective untreated control dish consists the time-point (incubation day) of maximum Increase of Diameter Effect (T_{IDE}). The value of the Increase of Diameter Effect-IDE is recorded in the last column and is expressed as per cent (%) increase of colony diameter (or of the respective average where applicable) compared to the control(s) on the T_{IDE} day.

An exception to the kinetic nature of all the abovementioned cases of stimulation was possibly observed with the *Mucor* sp strain UOA/HCPF 11596 in MEA, treated by 0.5 mA under exposure pattern (ii). There was no sign of reaching a plateau in the proceedings of the experiment (until the Petri dish was fully covered), and thus the difference seems to be Dynamic in nature (as defined in the Discussion section), but this is inconclusive: Longer incubation of the culture (impossible due to the size of the dish) might have produced a plateau at the level of the Control, or not.

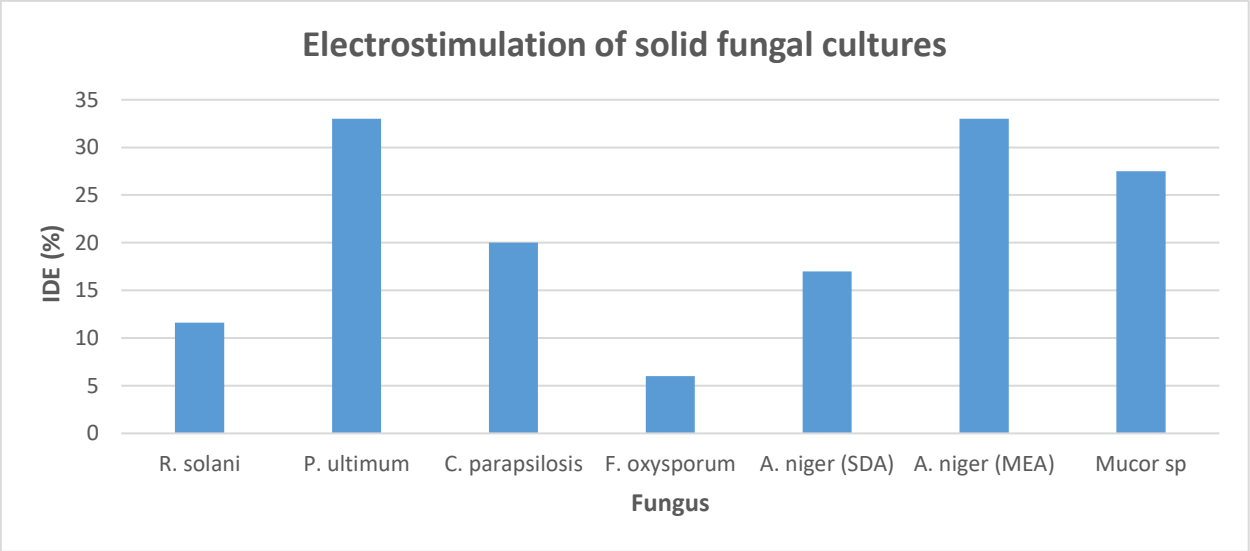

Figure 4. The inductive effect of ES on the tested fungi, according to data of **Table 2**, expressed as Increase of Diameter Effect-IDE, the percent increase of the mycelial /colony diameter of the sample (s) compared to the untreated control (s).

The same fungus in MEA is much more affected than in SDA, and less evolved fungi seem to be more affected than more evolved ones in the same substrate (see **Table 2**).

4. Discussion

The phrase "Kinetic difference" that has been used throughout the Results section implies herein that the strains presenting it have the same plateau, in terms of population growth metrics (viable counts, colony/mycelium diameter in solid cultures, absorbance in liquid cultures) but reach it in different times after inoculation. Thus, the difference is temporal, not quantitative. It may also be a matter of different slopes at the curve; the difference being neither quantitative nor temporal, but qualitative. It is possible to have both qualitative and temporal differences between strains presenting *kinetic difference* in growth. The *dynamic difference*, on the other hand, is quantitative. It occurs when the same phases, such as the plateau of the growth curves of two compared cultures/strains, differ in terms of growth metrics (including, but not limited to biomass assessment, colony diameter, Optical Density-OD readings). For example, the –prospective- eventual coincidence of the growth plateau for samples treated with exposure pattern (i) and untreated controls of the *F. oxysporum* UOA/HCPF 14172 strain [26] suggests that the observed difference was kinetic, not dynamic in nature.

Different results with other mycelial fungi under similar conditions make obvious that species peculiarities are important (see **Table 2**). The same can be concluded for dosage, as the intraspecies experiments show different growth rates by intensity and exposure pattern (**Table 2**); the latter being a measure of accumulative dosage, as mentioned before [26]. The considerable number of fungi (six-6) that showed stimulatory results in different conditions suggests a broad but variable biological basis of the electroinduction, in which the substrate seems to have a role, as the *A. niger* strain UOA/HCPF 10603C presented almost double the induction rate in MEA than in SDA (**Table 2**).

It is entirely possible that all fungal species are amenable to ES induction under some set of conditions or other. This set is expected to be characteristic of species, strains or of specific phenotypes in the case of pleomorphic fungi [52].

To screen for indications of inductive effect in the widest possible range of the relevant parameters, an expanded panel of parameters was tested in this study: Nature of stimulation, treated organisms and state of substrate. Bacteria were tested, in liquid cultures, with extreme frequency and amplitude settings and for different exposure durations. Additionally, the comparison was not only among differently ES-treated samples and untreated controls. Controls treated with a conventional thermal pasteurization protocol, which is considered as minimal thermal processing for heat-sensitive products, including but not limited to edibles, were compared to samples treated with the pasteurization protocol plus the range of ES settings that were used as unique treatment. Heat and ES were administered either simultaneously or consecutively. The objective was to either confirm the prerogatives of the Bioelectric Effect concept, which suggests that ES combined with other antimicrobial amenities result in synergy and increase in antimicrobial effect [11,53,54]; or to establish possible antagonism. The latter, should it be the case, might be explainable *inter alia* by the triggering of some heat shock response or similar cell mechanism, a very interesting feature for future research.

As a general observation there was no consistent pattern regarding induction maxima for Gram-negative bacteria. For the Gram-positive ones, an initial observation would be that the combination of the lowest settings, may result in induction if combined with the minimal exposure time (1 min). On the other hand, when the highest settings result in induction, this occurs at the 10 min exposure time. Induction has not been observed with the combined regimen applied consecutively.

The *x*-biotics, where "x" may be pro-, pre-, post-, meta-, pharma-, nutri- and any other conceivable prefix [13,15,17,55–57], seem as a collective pathway of intervention more naïve in both physiological and ecological terms, compared to usual drug-based intervention, especially regarding micromolecular, synthetic drugs [57]. The *x*-biotics are more compatible with Green concepts and Circular Economy [58,59] and draw upon extensive current and previous progress in the field of the Microbiology, in terms of microecology [60], microbial physiology [61], systems biology [62], metabolomics [23], and (auto) infectomics [63]. The *x*-biotics seem to hold great promise as a method to uphold and boost (or reboot) immunity [16,64] and also as a method to deliver drugs more elegantly and tolerably [65], or to enhance the efficacy and alleviate the adverse reactions of micromolecular/conventional drugs [66]. Thus the ramping up of production in terms of end biomass (dynamic improvement) or of time-to-end biomass (kinetic improvement) entails obvious economic interest for the production phase of *x*-biotics, in this timeline mainly probiotics and pharmabiotics.

Except the health dimension of probiotics, other, more direct applications of microorganisms of medical interest may be identified: Microbiotherapeutic strains [10], and strains producing biopharmaceuticals or other therapeutic amenities [7]. The food industry is a great prospective beneficiary [1,2] and thus possible sponsor to such research, while environment sustainment/protection/reclaiming would also find an interest [3] as the mass of respective strains used is huge and diverse and uprating

the production with no environmentally negative footprint sounds ideal. Last but not least, different fields of biotechnology [4], synthetic biology and xenobiology included [67], may benefit as well. The idea is based on the well-known though overlooked effects, both suppressant and inductive, of electricity on the microbial growth, both *in situ* [68] and *in vivo* [69]. Prospectively, if such approaches may be introduced into mass production of any microbial amenity (although this study is limited to live bacteria and fungi), it would magnify savings and flexibility by deleting or curtailing the need to produce and administer expensive and difficult to manufacture biochemicals into the batch culture process [70,71], which entail costs for production, storage and transportation, plus the associated ecological footprint of these procedures. Upon encouraging results, basic research, with many more organisms and exhaustive testing of electrostimulation settings and incubation conditions, would become feasible and of economic interest at a level alleviating the risk of funding. In this way it will be feasible to explore the vast potential of this approach, especially given that a much higher proportion of microbiota seems to be culturable than hitherto suggested [72], to a great extend due to the advent of Culturomics [73].

5. Conclusions

The use of electrostimulation or, rather, the effect of electric modalities on microorganism growth is nothing new [2,5,6] and has been long studied for treating liquid edibles [74]. Its use is considered mainly suppressive [74–77] but not exclusively so [1]; pilot experiments show important differentiation due to treatment settings, culture/incubation conditions, and among species, thus establishing the notion of electroculturomics [26]. Inductive effects can be achieved in very different conditions as proven by the pilot experiments presented herein, which were designed with the sole purpose to reconnoiter as wide a spectrum of potentially inductive settings and applicable culture formats as possible, for the purpose of informing future basic research. Thus, widely different conditions have been tried; solid vs liquid cultures; fungi vs bacteria of both Gram categories; unique vs repeated exposure patterns; exclusive ES treatment vs combined ES-pasteurization regimens (simultaneously or consecutively with the ES); AC settings nearing DC versus relatively extreme AC settings. In all cases, there has been indication that inductive effect may be expected.

This effect is in many cases kinetic rather than dynamic, meaning that the microorganism does not reach a higher growth/population maximum than if untreated, but that it reaches the maximum much faster, as is the case with the treated strain UOA/HCPF 14172 of *F. oxysporum* [26]. This implies that cultures may reach their full productivity in less time, increasing their profitability for both biomass and metabolite-production purposes, the latter inseparable from biotransformation/bioprocessing pipelines [78,79]. If metabolites are the intended product of the culture, as was the case with the Bioelectric Reactors—BER [69], these are usually produced during the steady, plateau phase, which, when under treatment, comes faster than it does spontaneously, saving time and curtailing costs (such as energy for the incubators). Prolonging the productive phase of the

organism/culture, is another beneficial prospect and it amounts at the very least to the temporal gain for reaching the growth plateau. The last proposal is only an educated guess and should be better studied in proper, full-scale electroculturomics studies [26,73,80,81]. If the intended product of the culture is biomass, the fast emergence of the maximum possible biomass production is even more desirable. Such cases include the preparation of commercial formulations for end users, meaning for direct consumer uptake/use, and also for industrial use, as is the case of additives to food products and of starter cultures used in bioprocessing edibles and beverages.

There is no uniform set of parameters for inductive results of electrostimulation, neither in bacteria nor in fungi. Different species show different developmental reactions to a given stimulus and, as a consequence, may undergo induction under different ES settings. It seems that some substrates are more supportive to stimulative effects of ES than others, as seems to happen with MEA compared to SDA in mycological applications. In any case, it seems that the optimization step for every single application/microbiote cannot be skipped and would require for its implementation an electroculturomics format, however plain and crude. Whether very similar species, with similar cellular and biochemical structure, form and profile (but not necessarily high genomic homology) would be squeezed under the same or similar set(s) of conditions, is one more subject for near-term further research. And a hot issue would it be, as it permeates the applicability of simplifying logistics for the use of closely related strains in ES-dependent applications, so as not to get engaged in issues of proprietary/patented /registered strains.

6. Future perspective

The transformation of this pilot study into basic research would allow to systematically test many more microbiota grown in diverse substrates and under various EM treatment conditions, or even modes of EM amenities (i.e. Electric fields/currents, Magnetic Fields), thus defining the field of electroculturomics [82]. The manipulation of microbial growth by EM amenities has a very wide field of applications, with suppressive and inductive applications. As this work focuses only on the latter, one may suggest that the most obvious mid-term iteration regarding the conduct of basic and translational research would be the exhaustive testing of the effect of EM with diverse culture media containing inhibitive concentrations of antibiotics of various compositions and mechanisms of action. The present work suggests that different microbiota present different windows of electrostimulation with beneficial effects, including the alleviation of effects of standard antimicrobial treatment. Thus, if such windows of EM treatment alleviate or nullify the microbicidal, or even microbiostatic effect of antibiotics one might hypothesize that combined regimens may be identified, where some microbial taxa would become less susceptible to the effect of different wide spectrum antimicrobial amenities, including but not limited to different classes of conventional antibiotics. The development of combined regimens that would protect to some degree the beneficial taxa of the appendage microbiomes from the effect of wide-spectrum antibiotics, as

the latter remain valuable in prophylaxis and empiric treatment. Such a development could re-iterate the use of existing, but also of future antibiotics.

The above would require either massive, extensive experimentation in high-throughput electroculturomics, or, alternatively, the use of predictive Artificial Intelligence-AI amenities. These would be, admittedly, of the Wide AI category rather than the more ubiquitous Narrow AI toolkits, as the latter might not be able to handle the number and changing priority of the conditions that affect the final results. Artificial Superintelligence would be preferable, but the affordability, safety and availability of such tools might hinder the progress in the field [83].

The long-term prospect is even more promising and regards microbiome intervention without any integrated use of antibiotics/antimicrobials. Given that different species show different developmental reactions to a given stimulus/set of stimuli (the stimulus is meant as nature of treatment, dosage, way of administration/application and number of treatment sessions), there is the possibility of precisely targeted effects *in situ*. This prospect is bolstered by recent achievements in remotely targeted ES [84], which follows the previous iteration, the contactless electrostimulation [85,86]. The bodyforms of most animals and plants, including humans, are practically transparent to electric and electromagnetic modalities [87], which is the operative principle behind MRI and CT scans. Thus, by using a given set of parameters, a specific member of the microbiome may be suppressed or induced [1]; perhaps not only one, but at least the one/ones that are needed to be affected. Such semi-surgical accuracy may be combined with oral or other administration of microbiome formulations, orally administered or in any other way, including local application and surgical transplantation [88]. Once the healthy microbiome is applied/ingested, then electrostimulation will assist it to develop faster and thus establish itself and exert its benevolent effect by expanding, or by producing some metabolite. This course can be used for select microbiota, which are determined as missing or are needed to re-equilibrate a dysbiosis [89,90] to symbiosis; for example, if the native flora is selectively depleted by a narrow spectrum antimicrobial commodity, antibiotic or other. This is a much more delicate and complex condition than the usual Antibiotic Induced Microbiome Depletion - AIMD [91]. But it may also be used in a wider context, by using a given cohort of stimulation parameters, to repopulate biocompartments that have been practically sterilized for medical reasons (i.e. before a surgery) or due to a pathogenic condition or accident, as in the case of extensive burns, which would otherwise encourage alien and possibly aggressive microbiota to establish colonization and, shortly thereof, infection.

Organ engineering and increased computing power and data science advances, including but not limited to AI, may allow high-fidelity simulations [92] for *in situ* electrostimulation of select microbiome constituents. Wide AI has completed similar tasks for designing and identifying biochemical antibiotics [93]. Regarding the extremely complicated gut microbiome environment [94], such approaches seem inescapable [95]. If this process results in actionable results, then the prebiotics concept would be expanded and the chemical equilibria that might plight the use of (bio)chemical prebiotic compounds, especially in patients with complicated and diverse pathological backgrounds, be partially resolved or rather side-tracked,

resulting in safer and more effective probiotic/pharmabiotic treatment [96], which is always a major concern [97,98].

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