Potential of atmospheric cold plasma for biofilm control in food processing

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IAFP European Symposium, 30th March 2017
RATIONALE FOR SEEKING NEW FOOD PROCESSING TECHNOLOGIES

30% CEREALS FOOD LOSSES
In industrialized countries, consumers throw away 286 million tonnes of cereal products.

45% FRUIT & VEGETABLES FOOD LOSSES
Along with roots and tubers, fruit and vegetables have the highest wastage rates of any food products; almost half of all the fruit and vegetables produced are wasted.

20% OILSEEDS & PULSES FOOD LOSSES
Every year, 22% of the global production of oilseeds and pulses is lost or wasted.

45% ROOTS & TUBERS FOOD LOSSES
In North America & Oceania alone, 5,014,000 tonnes of roots and tubers are wasted at the consumption stage alone.

20% DAIRY FOOD LOSSES
In Europe alone, 29 million tonnes of dairy products are lost or wasted every year.

30% FISH & SEAFOOD FOOD LOSSES
8% of fish caught globally is thrown back into the sea; in most cases they are dead, dying or badly damaged.

20% MEAT FOOD LOSSES
Of the 263 million tonnes of meat produced globally, over 30% is lost or wasted.
Plasma: an ionized gas consisting of atoms, electrons, ions, molecules, molecular fragments, and electronically excited species (informal definition)

www.geo.mtu.edu/weather/aurora/
STATES OF MATTER

SOLID
- Tightly packed, in a regular pattern
- Vibrate, but do not move from place to place

LIQUID
- Close together with no regular arrangement
- Vibrate, move about, and slide past each other

GAS
- Well separated with no regular arrangement
- Vibrate and move freely at high speeds

PLASMA
- Has no definite volume or shape
- and is composed of electrical charged particles

Each addition of energy creates a change in state:

SOLIDS → LIQUIDS → GASES → PLASMAS

Little or no order
What is Plasma?

- Plasma is an ionized gas consisting of free electrons, ions, reactive atoms, neutral molecules and photons.

![Images of solid, liquid, gas, and plasma forms of water]

- The plasma state can be reached by supplying sufficient energy (heat or electric power) to a gas or mixture of gases.
- Plasmas can be operated both at low and atmospheric pressure.

Dr. Ariël de Graaf
What Plasma do we use?

**Temperature**
- Thermal
- Non-thermal/Cold

**Pressure**
- Low pressure
- Atmospheric pressure
- High pressure

**Gas**
- Air
- Oxygen
- Helium, Argon
- MAP gas mixes

**Mode of generation**
- Microwave
- Radio frequency
- Corona
- Dielectric barrier Discharge

**Mode of Delivery**
- In Package / Contained
- In –Line or Tunnel array
- Plasma Activated Water
- Plasma Activated Liquids
- Plasma Activated Substances
- Plasma Deposition
What is in Atmospheric Cold Plasma?

<table>
<thead>
<tr>
<th>Plasma Generated Species</th>
<th>Density (cm(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide ((O_2^-))</td>
<td>(10^{10} - 10^{12})</td>
</tr>
<tr>
<td>Hydroxyl (OH(^.-))</td>
<td>(10^{15} - 10^{17})</td>
</tr>
<tr>
<td>Hydrogen Peroxide ((H_2O_2))</td>
<td>(10^{14} - 10^{16})</td>
</tr>
<tr>
<td>Singlet Oxygen ((^1O_2))</td>
<td>(10^{14} - 10^{16})</td>
</tr>
<tr>
<td>Ozone ((O_3))</td>
<td>(10^{15} - 10^{17})</td>
</tr>
<tr>
<td>Nitric Oxide (NO)</td>
<td>(10^{13} - 10^{14})</td>
</tr>
<tr>
<td>Electrons (e(^-))</td>
<td>(10^{9} - 10^{11})</td>
</tr>
<tr>
<td>Positive Ions (M(^+))</td>
<td>(10^{10} - 10^{12})</td>
</tr>
</tbody>
</table>

Reactive oxygen species

Reactive nitrogen species

UV radiation, energetic ions, charged particles etc.

Effects of Non-Thermal Plasma on Mammalian Cells. 2011 Sameer Kalghatgi et al.
HOW DOES ATMOSPHERIC COLD PLASMA WORK?

Adapted from Mai-Prochnow et al. 2014. International Journal of Antimicrobial Agents, 43(6), 508–517

Advantages

LOW TEMPERATURE, SHORT TREATMENT, UTILIZATION OF NON-TOXIC GASES, ABSENCE OF HARMFUL RESIDUES, REduced WATER CONSUMPTION, LOW ENERGY/COST & ACTIVITY AGAINST ALL TYPES OF MICROORGANISMS

From 2004 – 2012, European Union reported a total of 198 produce-associated outbreaks


<table>
<thead>
<tr>
<th>Type of pathogen</th>
<th>Vegetables</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Fruits</th>
<th></th>
<th></th>
<th></th>
<th>Total outbreaks</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Salad</td>
<td>Leafy</td>
<td>Tomato</td>
<td>Other</td>
<td>Sprouts</td>
<td>Berries</td>
<td>Melon</td>
<td>Juices</td>
<td>Other</td>
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<tr>
<td>Norovirus</td>
<td>15</td>
<td>26</td>
<td>1</td>
<td>9</td>
<td>0</td>
<td>55</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>108</td>
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<td>Salmonella spp.</td>
<td>8</td>
<td>12</td>
<td>1</td>
<td>3</td>
<td>11</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>40</td>
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<td>Escherichia coli</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>7</td>
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<td>1</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>Clostridium spp.</td>
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<td>1</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>1</td>
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<td>4</td>
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<td>0</td>
<td>3</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
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<tr>
<td>Bacillus spp.</td>
<td>2</td>
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<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>1</td>
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<tr>
<td>Other foodborne viruses</td>
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<td>0</td>
<td>0</td>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>9</td>
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<tr>
<td>Other microorganisms</td>
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<td>0</td>
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</table>

Adapted from Callejon et al. 2015. Foodborne pathogens and disease, 12(1), 32 – 38.
Microbiological and Food Matrix Challenges relevant to fresh foods – Why do these risks persist?

- Pathogens
- Spoilage
- Biofilms
- Spores
- Toxins
- Internalisation / Structural protection
Investigations at lab-scale

DIT 120 System – High Voltage System

Output voltage: 0~120kV
Applied voltage: 80kVRMS
In-package treatment

Example of plasma streaming during treatment of cherry tomatoes

Advantages of IN package treatment - retention of efficacy, Time for longer lived species to effect target, mitigates recontamination or cross contamination events
EFFECTS ON E. COLI IN LIQUID MODEL

- 7 log reduction in MRD after **20 s of Direct and 45 s of Indirect ACP**

*Interactive effects of mode of exposure, treatment time and post-treatment storage time, media composition, voltage levels and working gas*

- Atmospheric air
- High voltage level
- Post treatment storage 24 h

BACTERIAL BIOFILMS

- Microbes exist predominantly as biofilms
- Community of cells surrounded by a matrix of extracellular polymeric substances (EPS) that hold microbial cells together to a surface
- Enhanced tolerance to high concentrations of antimicrobial agents
- 80% of human infections are associated with biofilms (NIH)
- A major challenge in food, environmental, pharmaceutical industries and in clinical and healthcare scenarios

Adapted from Monroe D. 2007. PlosBiology, 5(11), e307, 2458 - 2461

**Treatment of biofilms**

**XTT assay**

**Plate count**

*P. aeruginosa* ATCC 27853 48 h biofilm

ACP treatment process parameters:

- **Voltage:** 80 kV RMS
- **Mode of exposure:** Direct/Indirect
- **Post treatment storage time:** 24 h
Treatment of biofilms

*P. aeruginosa* 48 h biofilm

ACP: Air, 80 kV, 5 min treatment, 24 h post treatment storage

Control | Direct ACP | Indirect ACP

**SEM**

- **Thickness**: 23 µm
- **Thickness**: 10 µm
- **Thickness**: 10 µm

**CLSM (SYTO9/PI)**

- **Green**: live cells
- **Red**: dead cells

Mechanisms of removal?

ACP against *P. aeruginosa* QS-controlled virulence factors and biofilm formation capacity

- Bacterial QS is a population density controlled cell to cell communication system
- QS is used by bacteria to coordinate the expression of several genes involved in virulence, biofilm formation and pathogenicity.
- QS inhibition - an alternative antimicrobial target??

What did we find? ACP was effective toward reduction of virulence factors:
- **Pyocyanin** – 60 s resulted in almost complete reduction
- **Elastase (Las B)** - 5 min reduced by ~ 50 %
- **Biofilm formation capacity was not reduced** - ACP did not influence the ability of *P. aeruginosa* to form biofilms
- **Cytotoxicity** (CHO-K1) - ACP treatment significantly reduced cytotoxic effect of *P. aeruginosa* supernatant

**ACP technology may play an important role in attenuation of virulence of pathogenic bacteria**

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Non-thermal Plasma Exposure Rapidly Attenuates Bacterial AHL-Dependent Quorum Sensing and Virulence

Padrig B. Flynn1,*, Alessandro Busetti1,*, Ewa Wielogorska1, Olivier P. Chevallier1, Christopher T. Elliott2, Garry Laverty1, Sean P. Gorman1, William G. Graham3 & Brendan F. Gilmore3

The antimicrobial activity of atmospheric pressure non-thermal plasma has been exhaustively characterized, however elucidation of the interactions between biomolecules produced and utilized by bacteria and short plasma exposures are required for optimization and clinical translation of cold plasma technology. This study characterizes the effects of non-thermal plasma exposure on acyl homoserine lactone (AHL)-dependent quorum sensing (QS). Plasma exposure of AHLs reduced the ability of such molecules to elicit a QS response in bacterial reporter strains in a dose-dependent manner. Short exposures (30-60s) produce a series of secondary compounds capable of eliciting a QS response, followed by the complete loss of AHL-dependent signalling following longer exposures. UPLC-MS analysis confirmed the time-dependent degradation of AHL molecules and their conversion into a series of by-products. FT-IR analysis of plasma-exposed AHLs highlighted the appearance of an OH group. In vivo assessment of the exposure of AHLs to plasma was examined using a standard in vivo model. Lettuce leaves injected with the nifR/lasR mutant PAO-MW1 alongside plasma treated N-butyryl-

Figure 6. Lettuce leaves after three days incubation with (a) PAO1, (b) PAO-MW1 only (c) PAO-MW1 with 10μM BHL:OdDHL and (d) PAO-MW1 with 10μM of 120 seconds plasma treated BHL:OdDHL. 16μl of a 10⁶ CFU/ml bacterial suspension was injected into the mid-rib of five lettuce leaves for each condition with leaves monitored daily for three days at 37°C.
Mechanism of action
SEM Analysis – PTST –sealed container

**E. coli & L. monocytogenes**

**E. coli ATCC 25922**

Control 1hr storage 24hr storage

**L. monocytogenes NCTC 11994**

Control 1hr storage 24hr storage
Cell Integrity

**E. coli ATCC 25922:** Direct; Indirect

**E. coli NCTC 12900:** Direct; Indirect

**L. monocytogenes NCTC 11994:** Direct; Indirect

Voltage: 50kV; Treatment time: 0~120s;
Post treatment storage time: 24hr

Han, L; Patil, S; Cullen, P; Keener, K; Bourke, P* (2014) Bacterial inactivation by Atmospheric Cold Plasma: Influence of process parameters and effects on cell leakage and DNA. Journal of Applied Microbiology. 116 (4), 784-794
More DNA damage in Listeria than E.coli

DNA damage effect of plasma.
Genomic DNA damage of (a) *E. coli* ATCC 25922; (b) *E. coli* NCTC 12900; (c) *L. monocytogenes* NCTC 11994
16s RNA PCR results of (d) *E. coli* ATCC 25922; (e) *E. coli* NCTC 12900; (f) *L. monocytogenes* NCTC 11994
Lane 1: Non plasma treatment control; 2: 5s directly treated samples; 3: 5s indirectly treated samples; 4: 30s directly treated samples; 5: 30s indirectly treated samples

Han, L; Patil, S; Cullen, P; Keener, K; Bourke, P* (2014) Bacterial inactivation by Atmospheric Cold Plasma: Influence of process parameters and effects on cell leakage and DNA. Journal of Applied Microbiology. 116 (4), 784-794
Intracellular ROS of G-/G+

Intracellular ROS levels in *S. aureus* were 3 times those in *E. coli* with same treatment time

**E. coli**

<table>
<thead>
<tr>
<th>treatment time (min)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>IF</td>
<td>8.1</td>
<td>7.5</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>OF</td>
<td>8.1</td>
<td>7.2</td>
<td>5.6</td>
</tr>
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</table>

**S. aureus**

<table>
<thead>
<tr>
<th>treatment time (min)</th>
<th>0</th>
<th>1</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus</td>
<td>IF</td>
<td>8</td>
<td>6.9</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>OF</td>
<td>8</td>
<td>6.5</td>
<td>6</td>
</tr>
</tbody>
</table>

Voltage: 80kV

Treatment time: 1, 3, 5 min

Post treatment storage time: None
Proposed Mechanism of action

(Han et al, 2016, Applied and Environmental Microbiology)

There is a Protection effect of food matrix

Han, L., Ziuzina, D., Heslin, C., Boehm, D., Patange, A., Millan-Sango, D., Valdramidis, V. P., Cullen, P. J., & Bourke, P*. (2016). Frontiers in Microbiology
Food based BioFilm Studies

Produce
Grains
Meat
Indirect ACP treatment of 70 kV reduced pathogens attached on produce surface and background microflora of produce

- **Effect of bacterial type:** ACP for 10, 60 s and 120 s eliminated *Salmonella, E. coli* and *L. monocytogenes* on tomatoes
- **Effect of produce surface characteristics:** extended treatment time was required for reduction of bacteria as well as background microflora on more complex strawberry surface

ACP was effective against biofilm populations:

- 5 min of treatment reduced biofilm populations on lettuce by $5 \log_{10}$ CFU/sample

**Effect of storage conditions for biofilm formation**: Temperature, light and time had interactive effects on bacterial proliferation, stress response and susceptibility to the ACP treatment.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Room T in light/dark</th>
<th>4°C in light/dark</th>
<th>4°C in dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLSM</td>
<td>E. Coli XL10 GFP</td>
<td>Supported bacterial internalization inside stomata</td>
<td>Resulted in lower incidence of bacterial internalization</td>
</tr>
</tbody>
</table>

Salmonella 48 h biofilms formed on lettuce at RT, light/dark
ACP: Air, 80 kV, 5 min treatment 24 h post treatment storage at 4°C

- **Importance of maintenance of the appropriate storage conditions** *(low T°C, minimised light exposure)* throughout distribution chain for the assurance of microbiological safety of fresh produce

- **Importance of effective microbiological control** as microorganisms protected by biofilms or complex structures of different produce commodities may present major risks of cross-contamination of the environment in food production sites
Bacterial biofilm formation in cereal-based media

- **E. coli**
  - NCTC 12900: strong
  - ATCC 25922: moderate
- **Bacillus ssp.**
  - B. atrophaeus var. niger, NAMSA: strong
  - B. subtilis ATCC 6633: strong
- **Lactobacillus ssp.**
  - L. plantarum ATCC 8014: strong
  - L. brevis ATCC 8287: strong

**Strains classification**:  

<table>
<thead>
<tr>
<th></th>
<th>E. coli NCTC 12900</th>
<th>E. coli ATCC 25922</th>
<th>B. atrophaeus var. niger, NAMSA</th>
<th>B. subtilis ATCC 6633</th>
<th>L. plantarum ATCC 8014</th>
<th>L. brevis ATCC 8287</th>
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<td>Standard medium (TSB or MRS)</td>
<td>strong</td>
<td>moderate</td>
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<td>Wheat model medium</td>
<td>moderate</td>
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<td>weak</td>
<td>strong</td>
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</tr>
<tr>
<td>Barley model medium</td>
<td>moderate</td>
<td>strong</td>
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<td>weak</td>
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</table>

Effect of ~80 kV of direct direct/indirect ACP treatment for 5 min on bacterial biofilms – colony count assay

Despite of weak to moderate biofilm formation in wheat and barley model media, ACP treatment efficacy against of *B. atrophaeus* was low.
Sporulation within *Bacillus* spp. biofilms formed in cereal-based media

It was determined that the 72 h biofilms of *B. atrophaeus* constituted on average 90% of spores using either wheat or barley model media for biofilm formation.
Effect of ~80 kV of direct direct/indirect ACP treatment for 5 min on bacterial biofilms – XTT assay

a) WHEAT MODEL MEDIUM

b) BARLEY MODEL MEDIUM
Inactivation of *B. atrophaeus* spores on abiotic surfaces that mimic grain surfaces

**Treatment** - 30 min reduced spores on hydrophobic surface by 6 log.

Only 4.2 log reductions were achieved with spores attached to hydrophilic surface.

Optical and electron microscopy showed physical changes of spores following ACP.
Inactivation of *meat spoilage* bacterial biofilm

Effect of ACP on *B. thermosphacta* 48h biofilm in 12% beef extract, treated at 80 kV (24 PTST) and assessed using plate count and XTT assay. (■) ACP treated, (●) untreated biofilm control.

Meat decontamination - Shelf-life study

Cold Plasma Control of Background microflora populations on fresh and cooked meat surfaces

Lamb chop

Sliced turkey

Pork loin

Graphs showing the log10 CFU/g over time for Lamb chop, Sliced turkey, and Pork loin, with different colors representing control, treated, improperly sealed tray, and properly sealed tray conditions.
Plasma-activated Liquids

- 70% H₂O
- Cancer treatment
- Sanitizing agent
- Surface decontamination
- Wounds
- Food

70% H₂O
Antimicrobial efficacy – $\text{H}_2\text{O}_2$ and pH

- Antimicrobial efficacy is dependent on $\text{H}_2\text{O}_2$ and pH
- Neutralization of pH removes antimicrobial activity
- Re-acidification can not restore biocidal effect
Achieving reactive species specificity within plasma-activated water through selective generation using air spark and glow discharges

Peng Lu¹,² | Daniela Boehm¹ | Paula Bourke¹ | Patrick J. Cullen¹,³

¹ Plasma Research Group, College of Science and Health, Dublin Institute of Technology, Dublin, Ireland
² School of Aeronautical Automation, Civil Aviation University of China, Tianjin, China
³ School of Chemical Engineering, University of New South Wales, Sydney, Australia

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Funding information
Science Foundation Ireland, Grant number: 14/IA/2626; China Tianjin Research Program of Fundamental and Applied Technology, Grant number: 14JCYBJC43000

Plasma-activated liquids (PAL) attract increasing interest with demonstrated biological effects. Plasma exposure in air produces stable aqueous reactive species which can serve as chemical diagnostics of PAL systems. Here, we tailor aqueous reactive species inside plasma-activated water (PAW) through treating water with AC air spark and glow discharges in contact with water. Chemical probing demonstrated species specificity between two types of PAW. Spark discharge PAW contains $H_2O_2$ and $NO_3^-$, while $NO_2^-$ and $NO_3^-$ are generated in glow discharge PAW. Species formation in different PAWs have been discussed in terms of discharge mechanisms and liquid phase chemistry process. Species specificity can provide richer parametric spaces for producing PALs with controlled impact and dosage achievable by selection of discharges.
Selective PAW/PAL for distinct applications?

Not cytotoxic

Anti-microbial

Modified plasma device
70μM H₂O₂

Mechanism of action?
Cell wall/membrane?

http://www.acpfg.com.au
www.slideshare.net
Possible advantages of ACP for food processing

• Non-thermal - heat sensitive ingredients
• Development of “new” products (e.g., shelf-stable PHF)

• Control Spoilage and pathogens - Extend safe shelf-life
• Can be In –package – Mitigates against Recontamination

• Can be a dry process – no chemical residues
• Can be a wet process – longer term effects?

• Can be built into process or equipment
• Low energy requirement and portability
### Acknowledgments

**DIT Applied Plasma Research Group**

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
</tr>
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<tbody>
<tr>
<td>Dr Paula Bourke</td>
<td>Dr PJ Cullen</td>
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<tr>
<td>Dr James Curtin</td>
<td>Dr Daniela Boehm</td>
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<tr>
<td>Dr Dana Ziuzina*</td>
<td>Dr Peng Lu</td>
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<tr>
<td>Dr Vladimir Milosavljevic</td>
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<td>Dr NN Misra*</td>
<td>Dr Sonal Patil*</td>
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<td>Dr Carmen Bueno Ferrer</td>
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<td>Dr Lu Han*</td>
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<td>Dr Diva Almeida*</td>
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<td>Miroslav Gulan</td>
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<td>Juan Perez</td>
<td>Roseane Cavalcante</td>
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**Prof Kevin Keener**

Iowa State University

BioCentury Research Farm

**Collaborating Institutions**

- National Centre for Plasma Science & Technology (NCPST)
- iris Research Engineering Technology
- Science Foundation Ireland (SFI)
- SEVENTH FRAMEWORK PROGRAMME
- Department of Agriculture, Food and the Marine
- Enterprise Ireland
- DIT