Chapter 8

Ultrasonic Decontamination and Process Intensification

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Abstract

Ultrasound is a versatile technology and has been successfully applied in several food processes including extraction, drying, decontamination, brining, mixing and homogenization, emulsification, freezing, thawing, and cutting of foods. High-power ultrasound can induce physical and chemical changes in the biological matrices due to mechanical, cavitational, and thermal effects. This chapter outlines the method and protocols employed in application of ultrasound for food applications. In particular, operation of contact and non-contact-type ultrasound systems with a main focus on microbial decontamination and process intensification (mainly brining of meat) is described in details. Various protocols for measuring ultrasonic process-product interactions including estimation of hydrogen peroxide and oxidation products are also discussed. Furthermore, methods evaluating antimicrobial effectiveness are described in detail.

Key words Decontamination, Process intensification, Brining, Hydrogen peroxide, Sodium chloride, Antimicrobial effectiveness

1 Introduction

Low-frequency ultrasound (US) employs high-intensity waves, which induce physical and/or chemical effects on food properties, and has a strong potential for a range of food industry applications [1]. The use of US for food applications can be characterized according to frequency and power, namely, low frequency/high power (<16 to 100 kHz and power from 10 to 1000 W/cm^2) and high frequency/low power (100 kHz to 10 MHz and power <1 W/cm^2) US [2]. Low-power US is mainly used in medical diagnostics, whereas high-power US is used in applications to induce physical and chemical changes in the biological matrices due to mechanical, cavitational, and thermal effects. Implosion of cavitational bubbles, formation of microjets, microturbulence, high-velocity interparticle collisions, and perturbation in microporous particles [3] result in enhanced mass transfer and accelerated chemical reactions [4]. Formation and collapse of cavitational

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bubbles generate extremely high localized temperatures (in excess of 5000 K) and pressures (500 atm) along with formation of free radicals [5] due to sonolysis of water ($H_2O \rightarrow OH^- + H^+$, $H_2O + OH^- + H^+ \rightarrow H_2O_2 + H_2$).

2 Ultrasonic Systems, Setup, and Characterization

The use of US for decontamination has been widely demonstrated on liquid foods and foods immersed in liquid media using contact-type systems (i.e., US bath and probe-based system) [6, 7]. Non-contact ultrasonic applications have been investigated in the food industry for defoaming [8], drying [9], and inactivation of microorganisms [10].

2.1 Contact-Type Ultrasound Systems

There are different variations of contact-type US devices in the market. US bath and probe systems are the most commonly used and can be applied to research microbial inactivation and process intensification (Subheadings 4 and 5). In an US bath machine, the US transducer is responsible for converting electric energy into mechanical vibrations and is typically located under the bottom of the tank. A transducer is usually connected to a probe or a horn which may be detachable, from which US is emitted into the medium. Usually, the probe system can deliver much higher power intensities than the bath system.

Most available power US systems are fixed at usually only one frequency; however, some systems can supply dual frequencies in one machine. Most machines supply operator control options for processing time, and some offer controls for power level or amplitude and/or operation mode. For a fixed frequency wave, larger amplitude means higher power. Some machines supply different operation modes: sweep, standard, degas, and/or pulse mode. In sweep mode, the frequency varies within a defined range, causing more homogeneously distributed ultrasonic efficiency in the bath than during standard operation. In degas mode, the power is interrupted for a short period so that the ultrasonic forces do not retain the bubbles. In some probe system, pulse mode is supplied, and the US irradiation on and off periods can be set so as to reduce the average power and heat generated in the liquid to a desired level.

Typical steps and protocols for contact-type US treatment of foods are described below:

1. *Sample handling and preparation*: The samples properties can be the key factor affecting US processing and its effect. Therefore, depending on the nature of material treated, the sample handling and preparation step in detail should be recorded.

2. *Preparation of US equipment*: Before operating the equipment, it is important to conduct a thorough pre-start check on the safety features of the power connection and the equipment setup. Switch on the generator before the US process to preheat the system followed by calibration as outlined below by system manufacturer. Most US devices are equipped with an intelligent automatic frequency tuning which ensures the operation of devices at correct frequency.
3. *Operational procedure for US treatment processes*
   (a) *Power measurement*: In order to standardize the intensity level of US process due to different US devices, ultrasound intensity (UI) for the used ultrasonic horn must be calculated. The UI can be determined calorimetrically using the following equations:

$$\mathrm{UI} = \frac{4P}{\pi d^2} \tag{1}$$

   where d [cm] is the diameter of the sonotrode.
   In the above equation, P [W] represents the absolute ultrasonic power and can be defined as:

$$P = mC_p\left(\frac{\mathrm{d}T}{\mathrm{d}t}\right)_{x=0} \tag{2}$$

   where m [g] is the mass, C_p [J/g K] is the specific heat capacity, and $\mathrm{d}T/\mathrm{d}t$ [K/s] is the ratio of change of temperature during sonication.
4. *US treatment*: Place the samples properly. Connect with external cooling circulation unit for temperature-sensitive material (if required) (*see* **Note 1**).
5. *Posttreatment analysis*: Retrieve the sample after the treatment and perform further measurements.

2.2 Airborne Ultrasound Treatment for Foods

Airborne ultrasound (AUS) utilizes non-contact transducers, which are capable of transmitting ultrasonic waves to a product using air as the coupling medium. Electrical energy is converted into mechanical vibrations in a piezoelectric transducer, which emits the ultrasonic waves through air toward the sample [11]. Ultrasonic waves may be transmitted by plate transducers by means of radiators with various shapes depending on the application as shown in Fig. 1. Plates are tuned to vibrate in flexural modes which can be circular, rectangular, or cylindrical depending on the application. The introduction of large plate surfaces has enabled an increase in the radiation resistance of air and resulting in an enhanced acoustic energy transfer between the vibrating system and the medium. The simplest plate radiator configuration is the flat-plate transducer which is characterized by a flat surface. The efficiency of this configuration

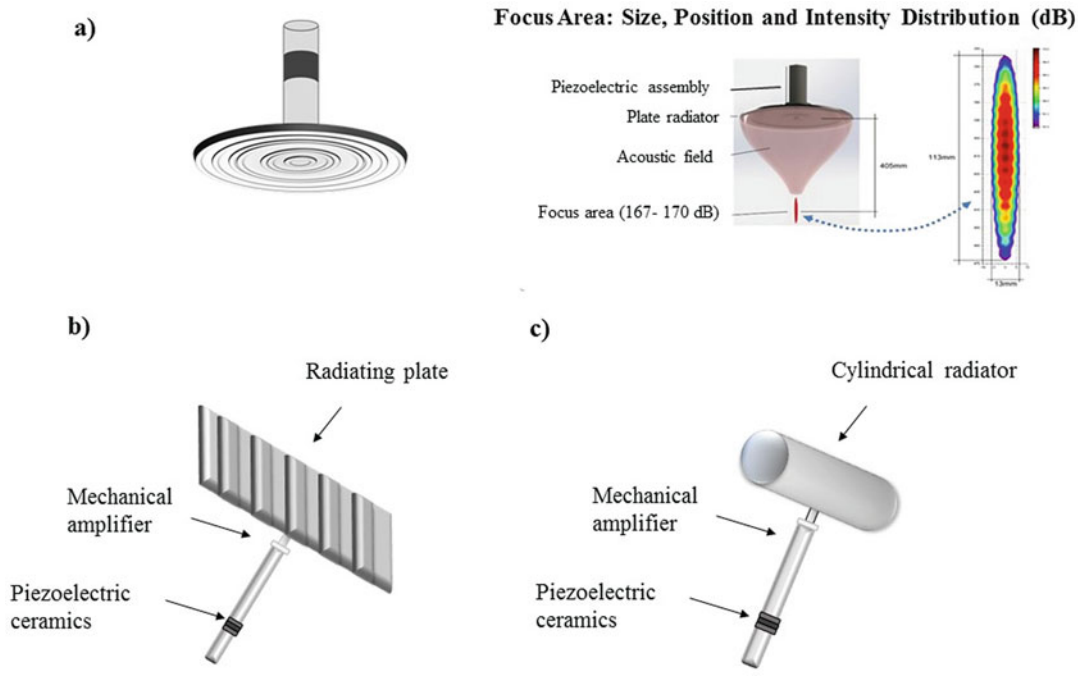

Fig. 1 Airborne acoustic transducers: (**a**) circular stepped-plate; (**b**) rectangular stepped-plate; (**c**) cylindrical plate (from [11])

is limited in the far field due to generation of lateral lobes in the acoustic pattern. The design of transducers has significantly improved recently enabling tailoring of the acoustic fields according to specific operational requirements. Highly directive coherent fields similar to those produced by a piston have been achieved by the addition of steps to the radiator surfaces, while steps and grooves have been shown to be capable of generating highly focused fields.

The efficiency and effectiveness of AUS processing is strongly dependent on the physiochemical properties of the food products used, sample preparation prior to processing, processing parameters, and the design of equipment. A typical system consists of an electronic power generator with a dynamic resonance controller, a power amplifier, a high impedance matching box, and a circular stepped-plate transducer. This type of transducer produces a highly focused acoustic field [12].

Typical steps and protocols for airborne US treatment of foods are described below:

1. *Sample handling and preparation prior to AUS treatment*: The samples properties can be the key factor affecting AUS processing and its effect; therefore, the entire sample handling and preparation steps should be recorded in detail.

2. *Preparation of AUS equipment*: Before operating the equipment, it is important to conduct a thorough pre-start check on the safety features of the equipment setups, as outlined below.
   (a) Mount the equipment safely by positioning it on a flat surface.
   (b) Ensure that the space where the equipment is located is well ventilated and there is no obstruction around the equipment.
   (c) Check the main power supply voltage and frequency to be connected to the equipment. Be aware of the location electrical safety symbols and electrical hazard warning signs of the equipment that can cause serious personal injury or equipment damage if ignored.
   (d) Check all the connections, especially the wire that connects the generator and the transducer (*see* **Note 2**).
   (e) Adjust the height of the system to fit the samples (*see* **Note 3**).
3. *Operational procedure for AUS treatment process*
   (a) Plug in the power meter.
   (b) Switch on the generator 20 min before AUS process to preheat the system.
   (c) Load the samples properly in the range of tailored hood (*see* **Note 4**).
   (d) Put on ear defenders and switch on the amplifier.
   (e) Retrieve the sample after treatment and do measurements.
4. *Monitoring output voltage and current*: Once the process is done, the output voltage and current can be obtained by the power meter.
5. *Evaluating the effect of AUS treatment*: It is crucial to determine whether the applied AUS treatment affect the cell microstructure and porosity. As a result of AUS treatment, the food sample is expected to experience subtle damage. Cárcel, García-Pérez [13] and Ozuna, Álvarez-Arenas [14] reported airborne US-assisted convective drying could generate microchannels in their apple samples, caused by "sponge effect."
   (a) Direct measurement of US intensity.
   (b) Estimation of the qualities of the samples: Freeze dry and store the representative specimens before and after AUS treatment. Follow the specific protocols for physiochemical analysis.
   (c) Microstructural analysis (suitable for solid foods).
      - Prepare the representative specimens before and after AUS treatment.

- Employ microscopic imaging techniques such as light, fluorescent, electron (SEM, cryo-SEM, TEM), or optical coherence tomography technique to identify the structural changes.

(d) Total viable count measurement: Prepare the representative specimens before and after AUS treatment, and dilute with maximum recovery diluent (MRD). Use pour plate or spread plate method to inoculate the samples on plate count agar (PCA) (*see* Subheading 4.1).

3 Ultrasonic-Induced Chemical Reactions

Ultrasonic cavitation causes the formation of gaseous nuclei within a liquid, with the subsequent disintegration of these bubbles [15–17]. Cavitation can be classed as stable and transient cavitation. In stable cavitation, these bubbles can persist for many acoustic cycles and are formed at low ultrasonic intensities (1–3 W/cm^2). In contrast, bubbles produced during transient cavitation will only survive a few cycles before violently collapsing due to higher intensities (>10 W/cm^2). This violent collapse results in the generation of high pressures and temperatures (>50 MPa and >3000 K) at the gas-liquid interface, which allows for the homolytic fission of molecular bonds. Most notably in aqueous environments is the breakdown of OH bonds in water molecules to form hydrogen (H•) and hydroxyl (HO•) free radicals.

The assessment of free radicals has attracted a lot of interest from the medical fields as these are involved in various biological process [18]. Their generation post-US treatments within in vivo and in vitro environments has been extensively studied [19, 20]. This allowed for the development of a variety of techniques to investigate their interactions [21]. The effect of free radicals within foods has been studied and focus on endpoint assessments that investigate changes in major nutritional compounds such as lipids, protein/amino acids, and vitamins [22].

The formation of free radicals during US treatments of food products can have positive impacts on their resultant quality as it acts as a decontamination process, thus improving their preservation [23, 24]. Still, the excessive formation of free radicals could result in adverse quality effects [25]. Here the formation of reactive oxygen species (ROS) would cause chemical changes in various compounds within these substances, potentially diminishing their nutritional and sensory properties. During US treatments, the formation of hydrogen peroxide (H_2O_2) as a long-lived ROS is well known and involved in the previous indicated processes [26]. Assay procedures that can be used to quantify H_2O_2 generated using titanium sulfate and substance oxidation using ferrous bound xylenol orange post-US treatment are outlined here.

3.1 Procedure to Determine Hydrogen Peroxide Generated Using Titanium Sulfate Assay

This assay involves the preparation of titanium sulfate, $TiSO_4$ reagent [27], that specifically reacts with H_2O_2 to form pertitanic acid (H_2TiO_4) that produces a yellow color. The generation of pertitanic acid can be assessed by measuring its absorbance at 410 nm. The following procedure is designed to use 1 cm quartz cuvettes with the appropriate spectrometer but can be adapted for 96-well plate readers.

1. Preparation of $TiSO_4$ (Eisenberg) reagent.
   (a) In a fume hood, prepare a sand bath and ensure that hot plate temperature is set at 150 °C.
   (b) With a 500 mL round bottom flask glass volumetric flask, add 200 mL of 96% sulfuric acid (H_2SO_4, CAS no.: 7664-93-9) and transfer 2 g of anhydrous TiO_2 (CAS no.: 13463-67-7). Leave the reaction flask for about 15 h at 150 °C.
   (c) Once complete, leave the mixture to cool to room temperature. Store the mixture in at room temperature and is stable for at least 6 months.
   (d) In a fume hood, prepare the $TiSO_4$ reagent by diluting 70 mL of the previous mixture in 210 mL of distilled water (H_2O).
   (e) Using a grade 4 (pore size $\leq$16–40 μm) or higher grade sintered glass funnel and a vacuum filtration setup, filter the diluted solution. The resultant filtrate is the assay detection reagent. This can be stored at room temperature for later use and is stable for at least 6 months.
2. Preparation of the H_2O_2 standard curve is done by diluting 30% (wt.) H_2O_2 (CAS no.: 7722-84-1) to make a stock solution of 100 ppm. This stock solution is then used to prepare 0.0, 5.0, 10.0, 15.0, 20.0, and 25.0 ppm standards. The standard curve can be adapted to represent better the test range being investigated.
3. Standard and sample analysis is done as follows:
   (a) Transfer 5 mL of standard or sample and add 500 μL of reagent.
   (b) Briefly vortex the resultant solution and incubate for 5 min at room temperature.
   (c) Measure absorbance at 410 nm for each solution in duplicate.
   (d) Using the standard curve, determine the concentration of H_2O_2 in each sample. The limits of detection and quantification should be determined as well.

3.2 Procedure to Determine Substance Oxidation Using Ferrous Oxidation in Xylenol Orange (FOX) Assay

This assay involves the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions that bind to xylenol orange molecules, generating a ferric complex that produces an orange color [28]. The formation of the resultant complex can be assessed by measuring its absorbance at 560 nm. The following procedure is designed to use 1 cm quartz cuvettes with the appropriate spectrometer but can be adapted for 96-well plate readers.

1. Preparation of 2.5 M H_2SO_4 is carried out in a fume hood. This is done by diluting 96% H_2SO_4 (CAS no.: 7664-93-9) in distilled H_2O to obtain the required concentration.
2. To prepare 25 mM ammonium ferrous(II) sulfate in 2.5 M H_2SO_4 (reagent A):
   (a) In a 100 mL glass volumetric flask, transfer about 50 mL of 2.5 M H_2SO_4.
   (b) Weigh 0.9804 g of ammonium ferrous(II) sulfate hexahydrate (CAS no.: 7783-85-9) and transfer this to the flask.
   (c) Top up with 2.5 M H_2SO_4 until the 100 mL mark and mix until all of the solute has dissolved.
3. To prepare the 100 mM sorbitol and 125 μM xylenol orange solution (reagent B):
   (a) In a 250 mL amber glass volumetric flask, transfer 100 mL of distilled H_2O.
   (b) Weigh 4.5545 g of D-sorbitol and transfer this to the flask.
   (c) Weigh 0.0224 g of xylenol orange and transfer this to the flask.
   (d) Top up with distilled H_2O until the 250 mL mark.
4. Reagents A and B must be stored at 4 °C and used within 1 month.
5. To prepare the assay detection reagent,
   (a) Mix 1 volume of reagent A with 100 volumes of reagent B by transferring 100 mL of reagent B and adding 1 mL of reagent A.
   (b) Mix the resultant solution (*see* **Note 5**).
6. Preparation of the H_2O_2 standard curve is done by diluting 30% (wt.) H_2O_2 (CAS no.: 7722-84-1) to make a stock solution of 100 ppm. This stock solution is then used to prepare 0.0, 0.7, 1.4, 2.1, 2.8, and 3.5 ppm standards (*see* **Note 6**).
7. Standard and sample analysis is done as follows:
   (a) Transfer 4 mL of assay detection reagent and add 100 μL of sample or standard.
   (b) Briefly vortex the resultant solution and incubate for 20 min at room temperature.

(c) Measure absorbance at 560 nm for each solution in duplicate.

(d) Using the standard curve, determine the concentration of peroxide in each sample. The limits of detection and quantification should be determined as well.

3.3 General Considerations for $TiSO_4$ and FOX Assays

These assays offer a relatively straightforward endpoint analysis to assess ROS formation post-US treatments. The increases in absorption for these assays at their specified wavelengths can be correlated to increases in ROS generation after the various treatments. Still interferences have to be considered when analyzing food products post-US treatment [28–30]. Spiked samples should be assessed to determine antioxidant, reducing and interfering effects. Compounds released during US treatment might have absorb at the same wavelength and bias the measurements. Therefore, absorbance measures of treated samples without the addition of assay reagents should be performed and subtracted from the final absorbance. Particulate matter released during these treatments is another concern; centrifugation and filtration steps would need to be included to remove these interferences. When multicomponent mixtures are being assessed, interactions must be specifically attributed to the specific parts of these mixtures. Model solutions should therefore be included in these investigations [24, 31, 32]. These could consist of the major components or specific components of the test substance. The exclusion and inclusion of components would provide a better understanding of the ROS interactions occurring during US treatments.

4 Procedures for Ultrasonic Decontamination

In recent years, the use of ultrasonic technology in water and wastewater treatment, as well as ecological remediation, including sanitization, has received a lot of attention [33, 34]. US produces elastic vibrations and waves with a frequency of more than 15–20 kHz. At low intensities and for short periods, US can induce microbial function and proliferation, but at higher intensities, it destroys and inactivates microorganisms. US with a frequency of 20–100 kHz and a sound intensity of 10–1000 W/cm^2 can be used to disinfect water for a long time [35].

Sonication's disinfection potential in liquids is due to the phenomenon of acoustic cavitation, which is the formation and collapse of microbubbles in milliseconds, causing extreme temperature and pressure gradients [36, 37]. When these microbubbles burst, the surrounding atmosphere is subjected to extremely high temperatures and pressures. Extremely reactive radicals have been observed in these conditions. US can inactivate bacteria and

deagglomerate bacterial clusters due to the physical, mechanical, and chemical effects of acoustic cavitation [38].

To evaluate the antimicrobial effectiveness and mechanism of action of US systems, a variety of protocols can be used.

- Viable counts of microorganisms.
- Viable counts of different mutant cells.
- Transcriptomic study.
- Sequencing of the whole genome.
- Other methods, such as flow cytometry, comet assay, and so on.

4.1 Viable Counts of Microorganisms

Food safety quality standards are mainly oriented toward the intended use and are outlined in international guidelines and legal requirements. Many environmental factors such as temperature fluctuations, air, and light can spoil food and water while also providing a breeding ground for bacteria. Bacterial pathogens, including indigenous aquatic bacteria, viral pathogens, and protozoan parasites, are all present in the environment. US has been related to oxidative stress in *Saccharomyces cerevisiae* [39], *Francisella tularensis* [40], *Escherichia coli* [41], and *Listeria monocytogenes* [42].

Serial plate counting using conventional microbiology methods and species-specific media can be a quick way to detect microorganism reduction after US treatments.

1. Aliquots (1 mL) are collected from US treated solutions.
2. Serial dilutions of samples at different times are performed, and the appropriate dilution is spread on tryptic soy agar (TSA) plates for assessing natural microflora.
3. Samples are incubated at 30 °C for 48 h.
4. Low microbial population counts are assessed by plating 1 mL of the sample over three TSA plates according to ISO 7218: 2007.
5. Microbial counts are expressed as log CFU/mL (*see* **Note 7**).

4.2 Viable Counts of Different Mutant Cells

Microbial mutants may be used to study the antimicrobial mechanism of action of US. Mutants with particular genes knocked out, for example, can aid in determining the effect of US on different metabolic and biological factors within the cell. The role of the gene can determine the effect of the treatment on the cell, so in the case of knocking out certain genes, the effect on the cell can be determined by the treatment. After 3 min of continuous US treatments (200 W, frequency of 26 kHz), literature indicates that the mutants oxyR were more resistant to the treatment, while dnaK was almost as susceptible as the wild form [41]. According to Patil, Valdramidis [43], the soxR, soxS, oxyR, rpoS, and dnaK genes play

a significant role in the protection against reactive oxygen radicals. One of the phenomena induced by cavitation is the formation of radicals H• and OH•, as well as H_2O_2 [44], which is known to cause oxidative stress in bacteria [44]. The dnaK protein, in particular, is required for high-temperature growth and is involved in the regulation of the heat shock response. The heat shock response is a cellular response to a variety of stresses, including heat, ethanol, oxidants, and DNA-damaging agents, abnormal protein growth, viral infections, and nutrient deprivation [45]. The oxyR regulates the expression of the oxyR regulon, which consists of many genes. The oxyR protein, which is formed constitutively, is oxidized by H_2O_2. oxyR binds to target gene promoters in its oxidized form and activates transcription by enabling protein-protein interaction with RNA polymerase. In the cell's defense, oxyR-activated genes play both direct and indirect antioxidant functions, such as catalase's removal of H_2O_2 and the Dps protein's protection of DNA from oxidative attack [46].

Similar protocols of viable count assessments with all the aforementioned mutants can be performed following the previously described protocol.

4.3 Transcriptomic Analysis

Transcriptomics has recently been used to investigate the impact of various microbial stresses. Wecke and Macher coined the word "omics era" to describe this time in 2011. Our understanding of cell resistance mechanisms and/or regulatory networks that organize bacterial stress responses has improved as a result of studying gene expression through looking at RNA transcripts present in cells [47]. Several studies of the transcriptome of *E. coli* during such stress responses have added to our understanding of stress mechanisms (e.g., Chueca, Pagán [47]; Harcum and Haddadin [48]; King, Lucchini [49]; Royce, Boggess [50]; Yung, Grasso [51]; Zheng, Wang [52]; Li, Zhang [53]). Many antibiotics' modes of action, as well as mechanisms of bacterial adaptation and inactivation by heat or high hydrostatic pressure, have been identified in these studies [54].

In addition, transcriptional profiling has shown the activation of general stress responses and proteins following particular methods of treatment due to specific cross-resistance phenomena [47, 55, 56]. As a result, the first heat shock response in *E. coli* was identified in 1978 by Harcum and Haddadin [48]. They discovered that 20 proteins were heat sensitive and were influenced by transcription levels. This discovery ignited a flurry of others, revealing that certain heat shock proteins facilitate protein folding while others function as proteases [48]. Further research using RNA-Seq methods, which can capture the global transcriptional response during specific conditions in any organism, could allow for simultaneous analysis of all regions within the genome, as opposed to

other methods like RT-PCR, which are still limited to analyzing specific and recognized genomic regions.

The aim of conducting transcriptomic analysis is to determine which genes are turned on or off as a result of a treatment. It is typically vital to detect such differences right after treatment, but in most situations, in order to operate with viable cells, the bacteria may have reversed the differences that the US would have caused. Working with viable cells, on the other hand, means that any changes that occur inside the cell can result in long-term changes. Once the cells are isolated, RNA extraction can be carried out by a number of ready-made kits.

1. Check the QC of the extracted RNA by using bioanalyzers. If sufficient quality is achieved, the RNA library is prepared by attaching oligo-dT probes. This allows the poly-A RNA to be selected that produces the mRNA.
2. Break down the fragments with high temperatures to 200 bp log fragments, which are used to prepare cDNA libraries. At this point, the libraries are read on an analyzer to perform data analysis, called bioinformatics.
3. A number of software packages are available for such analysis. These software packages carry out a number of algorithms in order to compare the libraries extracted from the bacteria under study with reference genomes, found on databases. This type of analysis is carried out by preparing contigs from the samples studied.
4. Reference these contigs and annotate against the reference genomes downloaded from an online database.
5. Once these are compared, the packages would be able to export all the RNA genes expressed. The results can also be plotted visually by running volcano and PCA plots.

Venn diagrams and heat maps also visually express the genes obtained and easily show patterns between different treatments. These patterns would allow the researcher to search for specific genes in Gene Ontology databases and obtain specific pathways and mechanisms (KEGG).

4.4 Whole Genome Sequencing

Direct measurements of mutation frequencies are now possible thanks to whole genome sequencing [56]. This is particularly true in the case of *E. coli*, where 12 populations have been reported to spread independently for over 40,000 years [57, 58]. Microorganisms have been used to research adaptive evolution mechanisms because of their short generation times, large population size, accuracy of experiment results, and ability to maintain ancestor strains [59].

Pavlov and Ehrenberg proposed a model in which bacteria manipulate gene expression to rapidly respond to environmental changes in 2013 [60]. Their research revealed that bacteria can direct their resources toward cell growth by growing pathways that cause other sources of energy to improve metabolism rather than multiplying when faced with environmental stress [61, 62]. Ferenci [63] investigated this further and found a highly unbalanced relationship between stress and mutations in *E. coli*. As a result, cells in certain physiological conditions or carrying out specific mutations may be able to withstand non-thermal physical treatments [62, 64]. Studying the mechanisms of microorganism responses to US can aid in understanding the potential risks and preventing any potential safety accidents, such as induced resistance, uncontrolled mutations, and enhanced recovery. More research is needed to better understand how microorganisms react to US stress, such as oxidative stress systems, cell repair, and resistance regulation [62]. Furthermore, many scientists have been perplexed by the maintenance of genetic systems that seem to have no selective advantages. "Cryptic" genes, which have no role in wild-type organisms and require mutational activation for expression, are part of such a genetic system [65].

NGS analysis is carried out by using beads washed with extraction buffer and containing lysozyme and RNase A.

1. Once purification of the genomic DNA is completed, the libraries are prepared using manufacturer-specific library preparation kits.
2. This will allow the genome libraries to be sequenced by the analyzer.
3. The data obtained can then be analyzed by running BBDuk and preparing contigs.
4. These contigs are compared with the reference genomes, in order to find SNPs in the mapped data. These SNPs will give rise to mutations within the genome after treatment.

4.5 Other Techniques

Further studies in the gene expression may also validate or enhance the work performed in such research. Transcriptomic analysis is a very strong tool in the identification of gene expression; however such expression will easily revert back once the US stress conditions are not present anymore. For this reason, RT-qPCR tests can be carried out to confirm the significant transcriptional difference between genes in both treatment samples, using different strains. Specific genes can be selected to calculate fold changes between treatment and control samples as described by Gallup and Ackermann [65].

5 Ultrasonic Process Intensification

US process intensification makes the reactions and the production of materials more efficient by improving rates and the output yields. Ultrasonic can be used in a range of processes including diffusion, homogenization, dispersion and wet milling, emulsification, extraction, lysis, and sonochemical reactions. For instance, brining of the meat is one of the major technologies used in processed meat manufacture, as it enhances shelf life, flavor, juiciness, and tenderness of the products. However, the migration of NaCl from the brine to the meat matrix is normally quite slow [66, 67]. Power US treatment can modify cell membranes through cavitation, which can help with curing, marinating, drying, and tenderizing the meat tissue, therefore helping in the enhancement of food quality and safety profile of the products [68]. US can help to reduce brining time without significant negative changes in other characteristics of the meat such as changes in quality (color, texture, cook loss, expressible moisture), sensory attributes, oxidative stability, and microbial load [69, 70]. Moreover, power US, acting on meat texture and providing a better distribution of salt in the meat matrix, could be helpful in the development of reduced salt meat formulations [71].

5.1 Procedure for US-Assisted Brining

A typical procedure for brining of meat includes the following steps:

1. *Sample preparation*: Muscles are generally stored at 4 °C prior to being processed. The pH of all the muscles should be 5.4 ± 0.4 when recorded by direct insertion of a pH electrode along the length of the muscle. Before curing, the connective tissue is carefully trimmed from the surface of the meat.
2. *Brine preparation*: A desired concentration of salt solution is prepared (e.g., 15% (w/w) NaCl). Additional curing ingredients (sugar, preservatives, spices, etc.) can be added for certain kind of products.
3. *US system setup*: Brining of meat can be done either with a probe, bath system, or combination. Figure 2 presents research on US enhancement of salt diffusion in meat using both an US bath and a probe system. Meat is cured in brine solution in the US tank, with US irradiated from the bottom of the tank or from the probe from the top of the tank.
4. *Temperature control*: The temperature of the brine solution is maintained using an external cooling system, consisting of a refrigerated circulator, a heat exchanger, and a variable flow rate pump. Maintaining the temperature of brine is very important to minimize the thermal effect of US treatment.

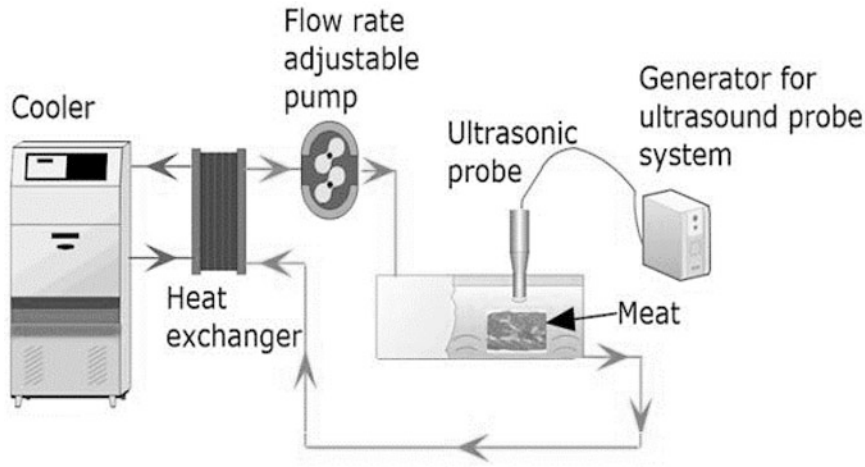

Fig. 2 Schematic diagram of experimental setup for brining of meat

5. *Brining*: Add desired brine solution in ultrasonic bath system at a desired frequency and treatment temperature. The ratio between the meat and the brine (e.g. 1:40 or higher) should be chosen to ensure no variations in the salt concentration of the brine between treatments.
6. *Further analyses*: After curing for sufficient interval, samples are rinsed with distilled water and stored for further analyses.

5.2 Method for Determination of Chloride Ion Concentration by Titration (Mohr's Method)

The Mohr titration is a direct titration method to quantitate chloride ions, to then calculate salt content. The method uses chromate ions as an indicator in the titration of chloride ions with a silver nitrate standard solution. After all the chloride has been precipitated as white silver chloride, the first excess of titrant results in the formation of a silver chromate precipitate, which signals the endpoint.

1. Prepare 0.1 M silver nitrate solution and standardize against primary standard KCl solution.
2. Weigh about 10 g of the sample and transfer it quantitatively to a conical flask, add 100 mL of hot water, and boil the content for 15 min with repeated shaking (*see* **Note 8**).
3. Filter the solution through glass wool. Transfer 50 mL of each solution to 250 mL Erlenmeyer flasks.
4. Add 1 mL of potassium chromate indicator to each 50 mL of filtrate.
5. Titrate the solution with standardized ca. 0.1 M $AgNO_3$, to the first visible pale red-brown color that persists for 30 s. Record the volume of titrant used.
6. Calculate the chloride content and the sodium chloride content in terms of percent and weight/volume.

5.3 Method for Determination of Sodium Content with Atomic Absorption Spectroscopy

Atomic absorption spectroscopy (AAS) method can be used for the determination of the concentration of specific minerals in foods. The limit of detection for sodium is up to 0.3 parts per billion (ppb). The ions in the sample solution are transformed to neutral atoms in an air/acetylene flame. Light from a hollow cathode or an electrodeless discharge lamp (EDL) is passed through the flame. The light absorption of the atoms in the flame, which is proportional to the ion concentration in the sample, is measured by a detector following a monochromator set at the appropriate wavelength.

1. Weigh approximately 10 g of meat and blend. Transfer into porcelain dishes, dry overnight, and burn on a hot plate.
2. Place the burned samples in a muffle furnace to ash at 525 °C for approximately 8–10 h.
3. Cool the ashes in desiccator and dissolve ash in few drops of hydrochloric acid (HCl). If necessary, filter the samples and make appropriate dilution of the samples (e.g., dilute to a volume of 50 mL) in a volumetric flask.
4. Sodium standard solutions were prepared using known concentrations of sodium chloride (NaCl) and calibration curves to correlate the relative absorbance.
5. Spike each standard, control, and sample 9:10 with 0.5% lanthanum chloride solution (one part of solution and nine parts standard, control, or sample) (*see* **Note 9**).
6. Follow manufacturer's instructions for startup, use, and shutdown of the AAS. Generally, the instrument should be recalibrated after every 20–30 samples. A control solution should also be run after each calibration.

6 Notes

1. Put on ear defenders and start the treatment at desired amplitude or power value.
2. Be careful with the wire that connects the generator and transducer because it is fragile.
3. The transducer and plate are very heavy. When operating the system, it is better to have two operators to adjust the system.
4. AUS is normally studied with the combination of other equipment such as fluidized bed dryer, plasma system, etc.; it is important to do trials to figure out the suitable sample conditions before the operation.
5. This volume will be enough to measure 12 standard and/or samples. It must be always prepared fresh on the day of analysis.

If more measurements must be carried out, prepare as necessary.

6. The standard curve can be adapted to represent better the test range being investigated.
7. Only counts between 30 and 300 CFU/mL are considered accurate measurements as they are easily read.
8. The salt can also be extracted from the food sample by means of ashing. Weigh the samples (10 g) into porcelain dishes, dry overnight, and place on a hot plate until completely burnt. Place the dishes containing the burned samples in the muffle furnace at 525 °C for approximately 8–10 h.
9. Lanthanum solution is added to prevent chemical and ionization interference.

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